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An investigation of the relationship between carriage of Leptospira and kidney disease in cats

An investigation of the relationship between carriage of *Leptospira* and kidney disease in cats

Submitted by

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Abstract

The aim of this study was to investigate *Leptospira* as a possible aetiology of chronic kidney disease (CKD), as well as investigating the relationship between demographic, haematological, biochemical, urinary and other variables, with leptospiral infection, in cats.

In this retrospective study, whole blood samples of 158 cats of the feline population of Langford Vets, Bristol, between September 2016 and October 2017, were analysed using a real-time polymerase chain reaction assay (qPCR) targeting the *lipL32* gene, present in pathogenic *Leptospira*.

When analysing the relationship between CKD and leptospiral positivity, 69 cats were excluded due to having existing conditions known or suspected to cause CKD. Of the remaining 89 cats, 2/33 (6.06%) of 'CKD' cats and 6/56 (10.71%) of 'non-CKD' cats were deemed leptospiral positive by PCR. There was found to be no significant association between CKD and leptospiral positivity.

All cats were included (n=158) when analysing the relationship between other variables and leptospiral positivity. 24/158 (15.19%) cats were deemed leptospiral positive. Statistical analyses revealed that leptospiral positive cats had significantly higher basophil and lymphocyte counts, as well as higher serum alanine aminotransferase. Leptospiral positivity was also significantly associated with AKI, current urolithiasis/nephrolithiasis, eosinophilia and lymphocytosis and biochemical hyperthyroidism. A negative correlation was found with age, abnormally low total protein and biochemical hypothyroidism.

Although, this study failed to demonstrate an association with leptospiral positivity and CKD, it was associated with AKI and younger cats. Younger cats may be more susceptible to infection by the bacterium, before an effective immune response is elicited, which then adapts to, and prevents, future infection. This initial infection may cause AKI and the damage caused later progresses into CKD as a cat gets older, despite the absence of active leptospiraemia by this point. Therefore, leptospiral infection may still be of aetiological relevance to CKD in the cats.

Dedication and acknowledgements

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Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Glossary

A:G	Albumin:Globulin ratio
AKI	Acute kidney injury
AP-PCR	Arbitrarily primed Polymerase chain reaction
CKD	Chronic kidney disease
CSF	Cerebrospinal fluid
Ct	Threshold cycle number
DJD	Degenerative joint disease
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FeLV	Feline leukaemia virus
FIV	Feline immunodeficiency virus
GFR	Glomerular filtration rate
gyrB	Gyrase subunit B
HIV	Human immunodeficiency virus
IgM	Immunoglobulin M
IRIS	International renal interest society
KD	Kidney Disease
LPHS	Leptospirosis pulmonary haemorrhage syndrome
MAT	Microscopic agglutination test
Non-CKD	Non-Chronic kidney disease
Non-KD	Non-Kidney disease
NSAIDs	Non-steroidal anti-inflammatory drugs
OMP	Outer membrane protein
PCR	Polymerase chain reaction
PCV	Packed cell volume
PKD	Polycystic kidney disease
qPCR	Quantitative Polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system

RI	Reference interval
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
USG	Urine specific gravity
UP/C	Urinary protein/creatinine ratio
VEGFR	Vascular Endothelial Growth Factor Receptor
UK	United Kingdom

1 Introduction

1.1 Feline Chronic Kidney Disease

Chronic Kidney Disease (CKD) can be defined as the 'structural and/or functional impairment of one or both kidneys that has been present for more than approximately 3 months' (Bartges 2012). Acute kidney damage or acute kidney injury (AKI), describes a condition that occurs suddenly, usually as a result of impairment of the tubular region of the kidneys and can be caused by certain nephrotoxic drugs or anaesthetic conditions (Grauer 2005), as well as urethral obstructions, dehydration and infectious causes (Ross 2011, Mugford, Li and Humm 2013) . If the resulting lesions are severe, it may lead to acute kidney failure and a poor prognosis. However, If the patient survives this acute insult, it can later progress to chronic kidney disease, as shown in one study in 99 dogs with acute renal failure (also known as AKI) (Vaden, Levine and Breitschwerdt 1997) in which 24 of the 43 that survived went on to develop a chronic kidney condition. CKD is predominately recognised as a geriatric disease amongst cats (Reynolds and Lefebvre 2013), with one study reporting the average age of cats diagnosed with CKD as 15 years (Bartlett et al. 2010). The underlying cause can initiate damage to any of the areas of the kidney in order for it to lose its function and once a large proportion of nephrons are non-functional, chronic kidney failure develops (Grauer 2005). Although also reported in dogs (Bartlett et al. 2010, Polzin 2013), it is recognised as the most common disease affecting the renal system in cats (Reynolds and Lefebvre 2013).

1.1.1 Diagnosis

Presence of azotaemia, continuous proteinuria and poorly concentrated urinary output on laboratory findings combined with the appropriate history and findings on clinical examination, is cause for diagnosis of CKD (Grauer 2015, Bartges 2012).

1.1.1.1 Clinical signs

Clinical signs indicative of the disease include polyuria/polydipsia, anorexia, lethargy, weight loss, vomiting, dehydration, constipation and halitosis (Acierno and Senior 2010, Reynolds and Lefebvre 2013, Elliott and Barber 1998, Greene et al. 2014). Studies report that anaemia is the most commonly seen consequential condition, especially in the later stages of the disease (Elliott and Barber 1998, King et al. 2007); this is thought to be due the reduced or absent production of erythropoietin by the kidneys. Loss of sight, secondary to hypertension caused by CKD can also occur (Acierno and Senior 2010), although this is thought to be rare with one study reporting blindness in only 1/80 cats with chronic kidney failure (Elliott and Barber 1998). One literature review acknowledged haematuria, difficulty urinating, abnormal faeces and neurological abnormalities as less commonly seen clinical manifestations (Reynolds and Lefebvre 2013). The earliest signs seen in cats is polyuria and polydipsia with one case-control study showing that this symptom developed in most in the year prior to diagnosis (Bartlett et al. 2010). Client education is important to enable prompt detection of the clinical signs associated with the disease in order to initiate earlier intervention, improving the prognosis (Paepe and Daminet 2013, Boyd et al. 2008)

Physical examination may reveal periodontal disease, as well as goitre, poor coat condition, cardiac murmurs, tachycardia and pale mucous membranes (Elliott and Barber 1998). In a retrospective case-control study, Greene et al. discovered that diagnosis of CKD was more likely in cats that had been reported to have periodontal disease or cystitis in the year prior to diagnosis (Greene et al. 2014). On palpation of the kidneys, either unilateral or bilateral reduction in size, has been associated with end-stage CKD (Elliott and Barber 1998).

1.1.1.2 Laboratory findings

Biochemistry, haematology and urinalysis tests can be performed to diagnose the disease, especially in the advanced stages (Paepe and Daminet 2013). Problems identified from these tests include proteinuria, elevated serum creatinine and urea, anaemia, isothenuria, metabolic acidosis, hyperphosphataemia, hypokalaemia, lymphopaenia, hypercholesterolaemia and microalbuminuria (Paepe and Daminet 2013, Lees et al. 2005, Bartges 2012). Elliot and Barber also reported increasing blood sodium levels and cholesterol levels, decreasing chloride levels and decreasing packed cell volume (PCV), with advancement of disease (Elliott and Barber 1998). Hypernatraemia was suggested to be due to a decrease in water intake as the disease progresses and the hypercholesterolemia due to disrupted lipid breakdown seen with kidney disease. Hypokalaemia can be common in the earlier stages, however, in the later stages, hyperkalaemia may also be evident due to the reduced or absent urinary output seen with severe cases (King et al. 2007).

Urine specific gravity (USG) is a measure of how concentrated the urine is and can be measured using refractometry (Watson 1998). A literature review stated that most cats with CKD will have isothenuric urine measuring between 1.007-1.015 although this may not be evident until the later stages of the disease (Paepe and Daminet 2013). In a study measuring USG in healthy non-azotaemic cats and azotaemic cats with CKD, it was found that it was significantly lower in the latter group with an average of 1.022 (Williams and Archer 2016). An Initial reading of USG on presentation has been found to have no significant association with survival time (King et al. 2007). It is also recommended to take into account the fact that this value is likely to undergo daily changes in any animal and unless it comes hand in hand with some of the other signs, it may not necessarily indicate CKD (Paepe and Daminet 2013). Some have argued, that due to findings in cats that have undergone renal ablation, USG is not an adequate marker of kidney function (Finch 2014). GFR measurement using renal clearance and plasma clearance methods may be a more effective measure of renal function (Finch 2014), although this is not as simple to implement in practice. Measurement of urinary protein: creatinine ratio (UP/C) has also been concluded to be an important factor in diagnosis, by predicting the level of proteinuria present with higher levels also being associated with shorter survival times (King et al. 2007).

1.1.1.3 Staging

Once diagnosed, CKD can then be staged according to the International Renal Interest Society (IRIS) guidelines (IRIS 2015). It is staged based mainly on the

presence and severity of azotaemia, although uses measurements of blood pressure and proteinuria for further sub staging.

Stage 1 refers to a non-azotaemic cat with a blood creatinine level of <140µmol/l with other mild renal abnormalities present such as poor urinary concentration. Stage 2 represents a mild azotaemia with a blood creatinine level of 140-250µmol/l with mild or no clinical signs present. Stage 3 cats suffer from moderate azotaemia with a blood creatinine level between 251-440µmol/l with the presence of more severe clinical signs. Cats with stage 4 CKD, also known as end stage, have a blood creatinine level above 440µmol/l.

Serum creatinine levels can also be useful for reflecting Glomerular Filtration Rate (GFR), although this value is also influenced by the muscle mass of an animal amongst other non-renal factors (Grauer 2015, Finch 2014). This should be considered when interpreting serum creatinine values, as well as urine specific gravity (USG) and physical findings to evaluate whether the azotaemia is caused by the kidneys alone (Grauer 2015).

Substages representing levels of proteinuria class non-proteinuric cats as having a urine protein to creatinine (UP/C) ratio of <0.2, borderline proteinuric (BP) cats as having a UP/C ratio between 0.2-0.4 and proteinuric (P) cats as having a ratio >0.4 (IRIS 2015). Increase in these values seen with progression of the disease are thought to be of consequence of glomerular lesions, with or without tubular lesions (Grauer 2015). Higher UP/C ratios are significantly associated with shorter survival times in cats with CKD (King et al. 2007).

Furthermore, arterial blood pressure can be analysed to further substage the groups by assessing risk of damage to the organs secondary to hypertension (IRIS 2015). Systolic blood pressure (mmHg) is <150, 150-179, ≥180, in minimal risk, low to moderate risk, and high risk substages, respectively.

1.1.2 Treatment

Although CKD is an irreversible condition (Barber 2003), evidenced based reviews available provide information on a multidimensional approach for the management of feline CKD (Roudebush et al. 2009, Korman and White 2013). These suggest that it is important to symptomatically treat to ameliorate the secondary conditions that commonly occur, such as dehydration, uraemia, hyperphosphataemia, hypertension, hypokalaemia, proteinuria, gastric symptoms, malnutrition and anaemia, to slow disease progression. This can be done so with dietary modification, medication, fluid therapy and various dietary supplements. It is also important to treat any concurrent disease such as diabetes mellitus, urinary tract infection, osteoarthritis, dental disease and hyperthyroidism, all of which are seen to commonly coincide with feline CKD.

Regular monitoring of the patient and prioritising treatment methods according to severity of symptoms, is important, as well as considering the quality of life of the animal. Although there is no cure, effective management can help prolong the life expectancy.

1.1.3 Aetiology of CKD

There are numerous possible causes of CKD in cats and they can be divided into two groups; congenital and acquired disease.

1.1.3.1 Congenital

Congenital causes of CKD include polycystic kidney disease, common in Persian cats and their related cross-breeds (Lees 1996, Greco 2001), which manifests as multiple cysts found in the kidneys that grow and interfere with renal function. One study found that 45% of Persian cats included had evidence of polycystic kidney disease (PKD), but actually found a higher prevalence amongst exotic cats (Beck and Lavelle 2001). Breeds such as the Exotic, Himalayan and Oriental cats are also at high risk of developing PKD (Rodriguez et al. 2014). Renal amyloidosis is a common inheritable disease in Abyssinian cats (Boyce et al. 1984, Lees 1996, Greco 2001), as well as Siamese, Oriental and Somali breeds (Lees 1996, Rodriguez et al. 2014). This is characterised by amyloid deposits within the kidney, commonly found in the medullary interstitium (Greco 2001).

1.1.3.2 Nephrotoxins and drugs

At the North American Veterinary Conference, clinically used drugs with potential nephrotoxic effects were discussed, and the management of the side effects evaluated (Papich 2005).Ones with the potential to cause harm to the kidneys include, tetracyclines, cyclophosphamide, cyclosporine, cisplatin, amphotericin B antibiotics, aminoglycoside antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs) and radiographic contrast agents.

Aminoglycoside antibiotics have been known to be a common cause of hospital acquired renal insufficiency in human patients (Nash, Hafeez and Hou 2002), as well as being nephrotoxic to small animals (Papich 2005). Their nephrotoxic effects may be due to the accumulation within the proximal kidney tubules which leads to disruption of the normal cell functions with consequential damage to the tubules (Papich 2005). However, one study analysing the effects of gentamycin on cats suggested that if given at the recommended daily dose, had little impact on renal function, but nephrotoxicity was more evident if this dosage was doubled (Hardy, Hsu and Short 1985).

The long term administration of NSAIDs can also have a negative impact on the kidney function of animals (Papich 2005). This is due to the inhibition of prostaglandin synthesis that results from these drugs, their role in tubular function and vascular tone is compromised and can cause kidney damage. However, it is thought that only those animals already suffering from renal dysfunction prior to administration would experience any of these negative side effects. One retrospective study found that NSAIDs such as meloxicam, commonly used to treat degenerative joint disease in cats, may not have nephrotoxic impact if administered at a lower median daily dose (Gowan et al. 2011). This study suggested that NSAID therapy may even slow the deterioration of a cat suffering from stable CKD, although it was unknown if this was due to direct effect on the kidneys or due to the alleviation of symptoms seen with the concurrent degenerative joint disease (DJD) occurring with the cats in this study. The patenty of its use, as with its salt derivatives, in the treatment of feline CKD is currently being applied for (Johnston 2011). Other drugs licensed in cats include Carprofen, ketoprofen, robenacoxib, and

tolfenamic acid and acetylsalicyclic acid, depending on region (Sparkes et al. 2010).

Lilies too, can have hugely detrimental effects on the kidneys of cats, with even small amounts eaten causing fatalities (Fitzgerald, 2010). This is thought to be a consequence of water soluble components targeting the tubular epithelium, being rapidly absorbed, causing acute kidney damage. Ingestion of ethylene glycol, commonly found in antifreeze solution, can lead to oliguric renal failure in cats, if not treated promptly (Balakrishnan and Drobatz 2013). The oxalates and glycolic acid formed by a cascade of reactions are the major contributors to acute tubular necrosis (Balakrishnan and Drobatz 2013). Persistent insult by these toxins or insufficient recovery after acute kidney injury could go on to cause CKD (Reynolds and Lefebvre 2013).

1.1.3.3 Viral

As seen with Human Immunodeficiency Virus (HIV), neuropathies have been associated with the feline lentivirus, Feline Immunodeficiency Virus (FIV) (Poli et al. 2012). When studying experimentally and naturally FIV infected cats, Poli et al. found that the histology and laboratory findings suggested the lentivirus to be a cause of the renal abnormalities. One study found that proteinuria was commonly found in cats infected with FIV compared to non-infected cats, although there was no association between azotaemia and FIV (Baxter et al. 2012). Others have found an association between the FIV and kidney dysfunction and although some have found a direct link (Poli et al. 1995) others questioned whether the FIV preceded the CKD or vice versa (White et al. 2010).

1.1.3.4 Hyperthyroidism

It has been reported that cats suffering from hyperthyroidism can develop azotaemic kidney disease after receiving treatment for the primary disease (Williams et al. 2010, Adams et al. 1997b, van Hoek et al. 2009). When exploring the mechanism for this, Williams et al. investigated the role of vascular endothelial growth factor (VEGFR), which was previously found to help maintain blood vessels and the renal vasculature (Williams, Elliott and Syme 2014). By monitoring its urinary excretion as a marker, this study concluded that although the hyperthyroidism indirectly leads to increased renal production of VEGFR (a marker of tubular hypoxia) via the renin-angiotensin-aldosterone system (RAAS), it was not associated with the development of CKD, and so the mechanism for the link between the two diseases remains unclear. Interestingly, CKD has been found to decrease the amount of total thyroxine concentration seen and conceal the effects of hyperthyroidism (Peterson and Gamble 1990) and in turn, there is evidence suggest that hyperthyroidism leads to a decrease in creatinine values via decreased muscle mass and increased glomerular filtration rate (GFR), leading to a misdiagnosis of CKD (Adams, Daniel and Legendre 1997a, Becker et al. 2000, Vaske, Schermerhorn and Grauer 2016).

1.1.3.5 Neoplasia

Another condition that causes specific renal lesions is lymphoma (Quimby 2015). Renal lymphoma was found in 10.6% of cats with organic kidney failure (Minkus et al. 1994) and is thought to be a potential cause of CKD in cats (Reynolds and Lefebvre 2013). A study in 118 cats diagnosed with

lymphosarcoma, 30% of the cases were found to have renal involvement (Gabor, Malik and Canfield 1998). A later report on the same cohort investigated the haematological and biochemical values of these cats (Gabor, Canfield and Malik 2000). Common findings in these cats with renal involvement showed evidence of anaemia, raised serum and creatinine levels, as well as azotaemia, being more significant in cats with renal associated lymphoma, which could be attribute to the ongoing renal dysfunction caused by the neoplasia.

1.1.3.6 Urolithiasis and nephrolithiasis

Urolithiasis refers to the formation of uroliths (calculi or stones) within the urinary tract (Gomes et al. 2018). Upper urinary tract uroliths are said to be associated with feline CKD (Reynolds and Lefebvre 2013). The majority of nephroliths (uroliths in the kidneys) and ureteroliths (uroliths in the ureters) are made of calcium oxalate (Berent 2011) and due the nature of this material, if it remains in the ureters, will lead to kidney damage. It is difficult to assess whether the cats with this condition had pre-existing kidney disease or if it is a primary cause of the renal damage (Reynolds and Lefebvre 2013, Kyles et al. 2005). One study found that 83% of cats with ureteral calculi presented with azotaemia (Kyles et al. 2005) and another found it in 95% of cats presenting with ureteral obstruction (Berent 2011), amongst other signs indicating impaired kidney function. However, in a case-control study, Ross and others found that there was no significant effect of the presence of nephrolithiasis on progression of mortality rates of cats with concurrent CKD (Ross et al. 2007).

1.1.3.7 Bacterial

Canine leptospiral infection can cause kidney damage of varying severity (Sykes et al. 2011, Levett 2001, Stokes and Forrester 2004) and this damage can persist to result in chronic kidney disease. With increasing evidence of leptospiral prevalence amongst cats (Section 1.2) and its similarity in clinical signs associated with CKD (Shropshire et al. 2015), it has become a subject of interest. Shropshire et al. performed the microagglutination test (MAT) on geriatric cats with stable CKD and geriatric cats without (Shropshire et al. 2015). It was found in this study that there was a significantly higher seroprevalence of leptospirosis amongst cats without CKD. However, it has been proven that PCR is a more effective diagnostic tool for the disease (Section 1.3) with there being greater limitations in using MAT. This study also only included 6 serovars belonging to *L.interrogans* species in the MAT, which, considering very little is known about the aetiology and pathogenesis of this disease in cats, is limiting to this investigation. In contrast, an earlier study used PCR on urine samples from healthy cats and cats with kidney disease of varying severity (Rodriguez et al. 2014). It was found that seroprevalence was significantly higher in the kidney disease group and, although there was a higher number of PCR-positive cats amongst the kidney disease group, this was not significant. However, the PCR was performed on urine samples with the G1 and G2 and B64-I/B64-II primers (Rodriguez et al. 2014). Although this PCR technique has previously been found to be effective (Bal et al. 1994), limitations in using these primer sets have also been reported (Gravekamp et al. 1993, Parma et al. 1997). There are other reported PCR techniques, found to be more effective in the diagnosis of leptospirosis (Section 1.3.5). There is

very limited evidence regarding the possible link between leptospirosis and feline CKD and there is need for further investigation using more up to date techniques.

1.2 Feline leptospirosis

Several reviews published recently have acknowledged the current lack of evidence surrounding feline leptospiral infection and its pathogenesis amongst this species (Schuller et al. 2015, Hartmann et al. 2013, Sykes et al. 2011)

1.2.1 Seroprevalence

In Serbia, despite there being no clinical signs of leptospirosis, out of 161 stray cats that were part of a neutering project, 26.7% were found positive for at least one serovar, the most common being Australis (then Pomona) (Obrenovic et al. 2014). Obrenović et al. also highlighted the variation in seroprevalence of feline leptospirosis in different studies undertaken in various geographical locations including France, Austria, Iran, Scotland and Brazil. Seropositivity ranged from 4.9-66.6%. The majority of studies available used MAT against serovars belonging to the *L. interrogans* species, commonly used in canine leptospiral diagnosis and it is evident that different geographical areas show a difference in common circulating serovar. However, due to the uncertainty that surrounds feline leptospirosis, MAT may be less relevant due to its specificity to canine infection and therefore it may prove useful to utilise a broader diagnostic tool allowing for deeper evaluation of infection amongst cats as there is clear evidence of other known pathogenic species, highlighted by one review (Evangelista and Coburn 2010), that could also be involved in feline infection. For example, when performing the MAT on the serum of stray and household cats in Iran (Jamshidi et al. 2009), the serovar Hardjo, Belonging to the *L. borgspetersenii* species, was included in the MAT and it was found to be the most common infecting serovar amongst the household cats tested. Another

variation amongst past studies, is the cut off value for a positive titre; some would deem a sample positive at a titre of 1:100 (Jamshidi et al. 2009, Lapointe, Plamondon and Dunn 2013, Markovich, Ross and McCobb 2012) whereas others may deem a titre positive when as low as 1:30 (Agunloye and Nash 1996). This would explain the range in seroprevalence amongst the different studies. Although some have implied that cats have leptospiral infection with a lower MAT (Markovich et al. 2012), when analysing canine leptospirosis, it has been suggested that using a higher minimum titre may be more beneficial for eliminating cross-reactivity between the serovars (Ellis 2010). Lapointe et al. suggested that an antibody titre of >1:200 may be more indicative of active infection (Lapointe et al. 2013).

1.2.2 Evidence of clinical disease

1.2.2.1 Clinical signs

There is a limited reported evidence of acute natural infection of leptospirosis in cats. Clinical signs classically associated with the canine infection may include anorexia, fever, lethargy, polyuria/polydipsia, weight loss and jaundice (Schuller et al. 2015, Sykes et al. 2011). In general, veterinarians tend to look out for clinical signs associated with renal/hepatic/coagulation deficits in order to make a differential diagnosis of leptospirosis amongst dogs (Shropshire et al. 2015).

Although in some cases, cats shown to be infected with the disease have not shown any clinical signs (Ullmann et al. 2012, Harkness, Smith and Fowler 1970), others have described signs similar to those presented amongst the canine species. Clinical manifestations of cases of suspected/confirmed feline infection include anorexia, weight loss, polyuria/polydipsia, vomiting, pyrexia,

lethargy and ascites (Agunloye and Nash 1996, Mason, McLachlan and King 1972, Arbour et al. 2012, Beaudu-Lange and Lange 2014, Reilly et al. 1994, Bryson and Ellis 1976), most of which would indicate renal insufficiency (Elliott and Barber 1998). Less common clinical signs reported include oral, pulmonary, cutaneous, ocular and reproductive adverse effects.

Ulcers and gingivitis (Arbour et al. 2012, Mason et al. 1972) have been reported in feline leptospirosis, although this can also occur in the presence of uraemia caused by renal disease in cats (Dokuzeylul, Kayar and Or 2016) and therefore could be consequence of kidney damage caused by the leptospiral infection, not secondary to the bacterial infection itself.

Arbour et al. also reported a chronic uveitis in one cat (Arbour et al. 2012), a manifestation that has been associated with the late phase of the disease in dogs, horses and humans (Townsend, Stiles and Krohne 2006, Faber et al. 2000, Chams et al. 2009).

Reproductive repercussions such as stillbirths and abortions have been reported in other species of livestock and domestic animals. One literature review highlighted a consensus that infection with Hardjo and Pomona may cause abortion in cattle and pigs (Levett 2001). The only case of this reported in cats was in 1994 when a queen delivered stillbirth kittens (Reilly et al. 1994). The livers of both kittens, as well as the placenta, demonstrated the presence the spirochetes, which may have been responsible for this reproductive failure.

Arbour et al. reported dyspnoea in one case of feline leptospirosis yet no *Leptospira* were found by bacterial culture of the lung tissue (Arbour et al.

2012). Post mortem examination of a cat in an earlier study revealed pulmonary haemorrhage and thrombosis in the lungs and diagnostic testing revealed presence of the spirochetes amongst this tissue. Similar findings have been reported in dogs and humans and thought to be due to leptospiral pulmonary haemorrhage syndrome (LPHS) (Kohn et al. 2010, Trivedi et al. 2010, Gouvela et al. 2008). Kohn et al. reported this in 70% of dogs with leptospirosis (Kohn et al. 2010) and another reported pulmonary changes by diagnostic imaging in 57% of canine cases (Knopfler et al. 2017).

An unusual case of feline leptospirosis occurred in France when a cat presented in a state of shock, with cardiac and respiratory abnormalities, as well as dehydration and weight loss (Beaudu-Lange and Lange 2014) and was found to be PCR-positive for *Leptospira* in blood. There was marked redness on the pinnae, as well as inflammation and a ‘marbled appearance’ to the skin on the foot digits and abdomen. Munday et al. reported the development of generalised cutaneous calcification in a dog over two months after its initial diagnosis of leptospirosis (Munday, Bergen and Roe 2005). These dermatological changes were hypothesised to be a consequence of a combination of the stress of systemic disease and renal dysfunction, leading to hyperphosphataemia, bacterial deposits in the skin and alteration to the dermal collagen. In the feline case (Beaudu-Lange and Lange 2014), a strong seroconversion to the serovar Saxkoebing, belonging to the serogroup Sejroë was reported by serology and it could be possible that this particular serovar could elicit this reaction. However, when beagles were experimentally infected with the same serovar, there were no clinical signs reported (Ruhl-Fehlert et al. 2000). It could therefore be possible that this response in the cat was either

specific to the individual host, or specific to cats, during infection with that particular serovar.

1.2.2.2 Liver involvement

Jaundice, suggesting hepatic impairment, has been reported in few feline case reports of suspected leptospirosis and one died, whilst the others were euthanased due to poor prognosis (Mason et al., 1972; Rees, 1964). Both authors described high titres to *L.* Pomona amongst these cases and it could be that this is a clinical manifestation of this particular serovar.

In contrast, Arbour et al. described three cats demonstrating a high titre to Pomona and showing no signs of hepatic dysfunction, but indications of acute kidney impairment, two of which fully recovered after treatment (Arbour et al. 2012). A cat found to be MAT positive for *L.* Pomona*,* despite being in good condition, was destroyed for investigation following an outbreak of human leptospirosis to a different serovar on the farm on which it lived (Arbour et al. 2012). Pale kidneys were noted on post mortem examination and histology revealed moderate interstitial nephritis as well as a generalised fatty parenchymal changes to the liver. This cat was thought to have become infected at its previous home on a farm where there had been an outbreak of bovine leptospirosis by *L.* Pomona six months previously.

When experimentally infecting cats with *L.* Pomona*,* Fessler and Morter reported a subclinical infection, after having not observed any clinical signs, yet noted all cats as having enlarged livers on post mortem examination (last cat

necropsied was at 61 days post infection) as well as varying degrees of microscopic hepatic lesions (Fessler and Morter 1964).

Collectively, these studies could suggest that feline leptospirosis has a long incubation period and jaundice could be a clinical manifestation of the very late stages of disease progression, particularly with this serovar.

1.2.2.3 Laboratory and other findings

In terms of laboratory findings of feline leptospirosis, Arbour et al. found results similar to that found in canine leptospirosis (Schuller et al. 2015), including elevated haematocrit, neutrophilia, elevated urea and creatinine, lymphopenia, thrombocytopenia and hyperphosphatemia (Arbour et al. 2012). On examination, all three patients had abnormally sized kidneys with altered definition and showed varied severity of renal insufficiency. Post mortem findings of other suspected/confirmed feline leptospirosis cases include interstitial nephritis, tubular nephrosis, hepatic and splenic amyloidosis, centrilobular necrosis or perilobular degeneration of the liver and haemorrhages of the brain and lungs (Mason et al. 1972, Bryson and Ellis 1976, Arbour et al. 2012, Rees 1964, Fessler and Morter 1964).

As the concept of feline leptospirosis is becoming more widely accepted, the use of up to date and efficient diagnostic tools is needed to further investigate its pathogenisis.

1.3 Diagnosis of leptospirosis

Leptospirosis presents with clinical and symptomatic similarities to other febrile illnesses and therefore can be confused with diseases such as Dengue or Malaria (Lindo et al. 2013). For this reason, a definitive diagnosis cannot be made from these observations alone. This disease also poses a threat to global health due to its worldwide distribution and potential to cause endemics (Sykes et al. 2011, Picardeau et al. 2014) and therefore it has been a necessity to develop an effective diagnostic tool to detect the disease at the earliest stage of progression to allow for rapid treatment and prevention of spread. Several reviews have evaluated the common findings found, and diagnostic techniques used, in leptospirosis (Schuller et al. 2015, Sykes et al. 2011, Ahmad, Shah and Ahmad 2005, Levett 2001).

1.3.1 Non-specific laboratory findings

Non-specific laboratory findings can be indicative of leptospirosis but would not be used to confirm infection. Investigations include haematology, biochemistry complete blood counts (CBC) erythrocyte sedimentation rate (ESR), white cell counts (WBC), liver function tests (LFT), urinalysis and CSF analysis, highlighted in one symposium and literature review (Ahmad et al. 2005, Levett 2001). Results commonly observed with the disease may suggest impaired renal function (with or without azotaemia), impaired hepatic function (Icteric leptospirosis), leucocytosis, electrolyte imbalances and possible anaemia (Ahmad et al. 2005, Levett 2001, Sykes et al. 2011, Knopfler et al. 2017) with thrombocytopenia also being a common finding amongst dogs (Knopfler et al. 2017, Kohn et al. 2010) . These findings may indicate infection, however do not

contribute to an aetiological diagnosis. Conventional diagnostic tests include direct microscopic demonstration, culture and serological tests.

1.3.2 Dark-field microscopy

Historically, diagnostic methods such as dark-field microscopy were used, in which blood or cerebrospinal fluid (CSF) were analysed in order to directly examine for the presence of bacteria in the samples (Levett 2001). Theoretically, due to the bacterial load in the blood during the acute stage (10^2-10^6 *Leptospira* per ml), it was thought to be an effective way of detecting the *Leptospira* (Picardeau et al. 2014, Agampodi et al. 2012). However, further use of the technique since has revealed its limitations. This method would not be effective during the immune phase due to the absence of bacteria in the blood and it has also been demonstrated to be open to misinterpretation with low sensitivity and specificity (Levett 2001, Musso and La Scola 2013), with one study performed on the blood samples of humans with suspected leptospirosis reporting the specificity and sensitivity of this diagnostic technique as 61% and 60%, respectively (Sharma and Kalawat 2008).

1.3.3 Culture

Isolation identification of leptospiral bacteria using culture techniques has previously been described as one of the gold standard methods of diagnosis (Picardeau et al. 2014). Either a urine, blood or CSF sample may be selected for culture but the presence leptospiral bacteria in each type of sample depends greatly on the stage of disease, making timings complicated (Ahmad et al. 2005, Musso and La Scola 2013). One meta-analysis of four separate studies in Thailand, evaluated the sensitivity of culture alone as being 10.5%

(Limmathurotsakul et al. 2012) in the diagnosis of leptospirosis. It has been admitted that this method has little relevance in early diagnosis of the disease (Toyokawa, Ohnishi and Koizumi 2011) due to the slow growth rate of the bacteria, prolonged incubation times and expertise requirements (Ahmad et al. 2005). Some have suggested that it would be a more effective diagnostic tool when used in combination with other methods (Tulsiani et al. 2011, Limmathurotsakul et al. 2012).

1.3.4 Serology

Serological tests have been found to be effective methods in diagnosing leptospirosis. This includes the microscopic agglutination test (MAT) and the IgM enzyme-linked immunosorbent assay (ELISA). The MAT is considered the reference method for serology (Musso and La Scola 2013) and involves mixing serial dilutions of patient sera with live antigens of known serovars and, once incubated, the samples are analysed under dark field microscopy in order to identify agglutinating clumps of *Leptospira* (Ahmad et al. 2005) . The highest serial dilution that shows agglutination with a minimum of 50% of the *Leptospira* is referred to as the 'end–point titre' and if this meets a certain cut-off value, the sample is identified as positive for leptospirosis (Ahmad et al. 2005, Ehsanollah and Gholam 2011). This method is limited due to the lack of a standardised cut off values amongst the different laboratories and those using lower values may allow for over diagnosis of the disease (Ahmad et al. 2005). The test is also vulnerable to misinterpretation, cross-reactivity, cross-contamination and the use of live antigens poses a health risk to personnel (Ahmad et al. 2005, Musso and La Scola 2013). This was demonstrated in the experimental infection with

Leptospira of young beagles in which MAT results would often showed highest titres to serovars differing from the ones purposefully inoculated into the dogs (Lizer et al. 2018), and this high level of cross reactivity was found in all serovars tested, except Hardjo. If the animal has recently been vaccinated, they may also show seropositivity to the serovars contained in the vaccine and may give false positive results (Martin et al. 2014). If the animal is infected with a serovar not present in the serovar panel of the test, this could in turn lead to false negative results (Sykes et al. 2011). Antibody production usually occurs after the leptospiraemic phase of the disease (Ahmad et al. 2005), during the second week of infection, and may not peak for 3-4 weeks and for this reason, this test may not be as effective in early diagnosis (van de Maele et al. 2008). Lizer et al. found seroconversion evident in 21/32 by day seven, later increasing to 30/32 by day 14 when using MAT (Lizer et al. 2018) when experimentally infecting dogs. Another time consuming factor that must be acknowledged concerning this method, is the common need for a fourfold titre rise in paired sera samples taken a few weeks apart, limiting use in acute cases of leptospirosis (Niloofa et al. 2015).

IgM enzyme-linked immunosorbent assay (ELISA) is similar to MAT, developed from the latter to be used as a simpler, more rapid screening test, but detects antibodies to whole cell *Leptospira* (Picardeau et al. 2014, Ahmad et al. 2005). It's specificity and sensitivity has been shown to vary and although it has been indicated to detect antibodies earlier than the MAT (Bajani et al. 2003), it gives no indication of infecting serovar (Picardeau 2013).

Due to the complexity of these more widely used serological tests, there was a need for a point-of-care test to allow for rapid diagnosis in practice. Recently, a lateral flow assay method detecting IgM antibodies has become available in Europe and studies have shown it may become an efficient method to promptly diagnose acute leptospirosis, with little effect of recent vaccination on result (Lizer et al. 2017, Lizer et al. 2018).

1.3.5 PCR

In recent years, polymerase chain reaction has become increasingly useful for diagnosing leptospirosis and tends to replace the serological techniques (Picardeau 2013). These methods target and amplify leptospiral DNA in samples in order to confirm presence of the bacteria (Harkin, Roshto and Sullivan 2003) and have been found to detect *Leptospira* prior to seroconversion (Brown et al. 1995), with studies detecting even small numbers of bacteria in blood and urine samples (Picardeau 2013). Real-time PCR assays using Taqman or SYBR technology have been found to be faster and more specific than regular PCR and so are more widely used (Picardeau 2013).

1.1.1.1 Targeting genes present in all bacteria

The *16S rRNA* gene (*rrs*), the *gyrB* gene and the *secY* gene are universally found in all bacteria (Ellis 2014) and have been targeted using PCR for detection of *Leptospira*.

One of the first target genes used in this method was that of the *L. interrogans 16S rRNA* gene (*rrs*) (Merien et al. 1992). Although the introduction of this

assay highlighted its sensitivity and speed superior to that of other methods such as culture, it was unable to distinguish between pathogenic and non-pathogenic species of *Leptospira* (Merien et al. 1992) and other studies since have also highlighted this shortcoming with positive PCR results reported for non-pathogenic strains of *Leptospira* when targeting this gene (Tansuphasiri et al. 2006, Thaipadunpanit et al. 2011). This method has also recently been found to amplify commensal or environmental bacteria in urine samples and so its sensitivity at detecting *Leptospira* alone was questioned (Fink et al. 2015).

The *gyrB* gene corresponds to the DNA gyrase subunit B protein and when evaluating a PCR assay targeting the leptospiral *gyrB* gene (Slack et al. 2006), there was no obvious amplification from the strictly non-pathogenic or pathogenic/intermediate species but it was present in all the known pathogenic species. Its topology in the species, *L. interrogans* and *L. kirschneri* was found to be distinctly different to that of the *16S rRNA* gene (*rrs*) on comparison, which could prove useful in species differentiation and could be explored further.

The *secY* gene is a housekeeping gene (Bourhy et al. 2011) and has been conventionally targeted in leptospiral PCR assays using the G1/G2 primers (Cheema et al. 2007, Gravekamp et al. 1993). Studies have found that its amplification is specific to pathogenic leptospiral species (Ahmed et al. 2009), however there is also evidence to suggest that the assays used to target this gene may not be able to amplify the DNA from some of the pathogenic *L. borgpetersenii* (Bourhy et al. 2011), *L. kmetyi* (Bourhy et al. 2011) and *L. kirschneri* (Gravekamp et al. 1993), as well as the DNA from the serogroup,

Grippotyphosa and Cynpteri, belonging to the species, *L. interrogans* (Parma et al. 1997). This proves to be a huge limitation, especially in terms of diagnosis in a clinical setting, and although some have used this set of primers targeting this gene in combination with others to detect pathogenic *Leptospira* (Cai et al. 2002, Gravekamp et al. 1993), it would be more desirable to have one set of primers that efficiently detected the pathogenic *Leptospira* alone.

The gene for OmpL1, a transmembrane outer protein (Haake et al. 2000), functions as a porin (Shang, Summers and Haake 1996). When developing primers to target this gene in leptospiral bacteria using arbitrarily primed PCR (AP-PCR), Reitstetter identified it in the majority, but not all, of the known pathogenic species (Reitstetter 2006). A study in China identified this gene in all the standard pathogenic strains as well as in 163 clinical strains of isolated pathogenic *Leptospira* (Dong et al. 2008). This study also found that the ompL1 gene could be divided into three subgroups amongst the standard strains and Reitsetter and Haake et al. found a divergence of the *ompL1* gene within the species, *L. interrogans,* splitting it into two groups (Reitstetter 2006, Haake et al. 2004). If explored further, these differences could prove useful in differentiating between pathogenic species and even serovars within the species.

1.3.5.1 Targeting outer membrane proteins

The genes for the outer membrane proteins, LigA and B, LipL21, LipL41 and LipL32 have all been investigated as target genes for leptospiral detection.

LigA and B are Leptospiral immunoglobin-like proteins suspected to be associated with leptospiral virulence (Matsunaga et al. 2003). Studies had shown primers targeting these genes to be specific to pathogenic *Leptospira* only (Bedir et al. 2010, Palaniappan et al. 2005). However, these investigations were limited to analysing pathogenic Leptospira belonging to *L. interrogans* and *L.borgpetersniia and L.kirschneri* and it would be useful to target serovars from other known pathogenic species to test overall efficiency. There is still little evidence surrounding this gene as a target for PCR assay, warranting further investigation.

LipL41 is expressed during infection and has been found to be pathogenic specific (Shang et al. 1996). Studies have confirmed that this gene can be found in many serogroups belonging to *L. interrogans* (King et al. 2013) and other investigations into this gene have highlighted its conservation amongst pathogenic leptospiral species as well (Shang et al. 1996, Natarajaseenivasan et al. 2005). When using the hamster model, there have been differing reports as to whether it is essential during infection (King et al. 2013, Barnett et al. 1999). Its questionable role during infection may hinder its efficiency as diagnostic tool and although some studies have advocated its use as an appropriate target gene for amplifying pathogenic *Leptospira* (Anitha et al. 2012, Shang et al. 1996, Natarajaseenivasan et al. 2005), further investigation is required.

The *lipL21* gene, which encodes another lipoprotein, has also been found to be a reasonably efficient target gene for PCR, although there is very limited literature available. However, it has been disputed whether it is present in some

pathogenic serovars such as the Ballum serovar, belonging to *L. interrogans* (Meenambigai et al. 2011, Cheema et al. 2007) or the *L.grippotyphosa* species (Meenambigai et al. 2011), which would limit its use as a general diagnostic tool in pathogenic leptospirosis.

The American College of Veterinary Internal Medicine (Sykes et al. 2011) and the British Small Animal Veterinary Association (Schuller et al. 2015) have both published consensus statements in the last eight years that highlight the efficiency of the *lipL32* gene (coding for a main outer membrane lipoprotein), also known as the *hap1* gene (Branger et al. 2005), as a target gene for PCR diagnosis of leptospirosis. Its expression during mammalian infection has been reported (Haake et al. 2000) and it has been associated with the development tubular nephritis in mice (Yang et al. 2002) . One study targeting this particular gene found the PCR assay to be 100% specific when analysing 218 laboratory strains of pathogenic *Leptospira* and showed no amplification in the non-pathogenic strains (Cermakova et al. 2013), agreeing with other findings (Levett et al. 2005, Stoddard et al. 2009). Levett et al. also discovered inhibitory effects on the PCR assay when analysing whole blood samples due to addition of certain anticoagulants and recommended the use of tubes containing Ethylenediaminetetraacetic acid (EDTA) or citrate anticoagulants only. When experimentally infecting 12 beagles, Branger et al. also concluded that targeting LipL32, produced a positive sample in all dogs infected by day 4 post inoculation, in the presence of leptospiraemia (Branger et al. 2005).

Many studies have experimented with various assays targeting this gene and there is very limited evidence available that draws any attention to any

limitations it may have. Some have proven a similarity in efficacy to targeting the *lipL21* and *secY* genes (Cheema et al. 2007). Whereas in bovine studies, the *lipL32* gene was amplified from all the pathogenic species studied in comparison to the *lipL21* gene, which could not be amplified in one (Meenambigai et al. 2011). Another study found Taqman and SYBR green assays targeting the lipL32 gene to be of superior sensitivity to those targeting the *secY* or *lfb1* genes (Bourhy et al. 2011) and when performing an endpoint dilution experiment, found that a SYBR green assay was of superior sensitivity to Taqman assays, when targeting lipL32. A very recent study further highlighted its use in diagnosing leptospirosis in the early stages of the disease, with its highest sensitivity in dogs with a lower MAT titre, which decreased as seroconversion progressed (Joseph et al. 2018).

As demonstrated, there are plenty of available genes to target when diagnosing leptospirosis and each study has highlighted their limitations and the need for further investigation. If these were indeed explored further, it is plausible that a sensitive assay could be produced for each one of the possible target genes. Due to the wider availability of literature promoting the use of the *lipL32* gene as a target for detection of pathogenic *Leptospira*, an assay targeting this gene was employed in the current study (Section 2.2.3).

1.3.6 Aims of this investigation

Due to the limited amount of information regarding leptospiral infection in cats and the possible long-term clinical affects this may have, the study aimed to investigate the presence of leptospiral infection as a possible cause of CKD in cats, using qPCR as a diagnostic tool. The only study available investigating this relationship in cats performed PCR on the urine only (Rodriguez et al. 2014). It was therefore decided to perform PCR analysis of *Leptospira* on whole blood samples in the current investigation to confirm an active leptospiral infection. It was also considered important to investigate possible effects leptospiral infection may have on the haematological, biochemical and urinary values in cats as well as to investigate the relationship between demographical details and leptospiral infection in cats.

2 Methodology

2.1 Case selection criteria

2.1.1 Origin of samples

Samples for the study had been collected from the feline surgical and medical patient population of the small animal referral hospital and small animal practice at Langford Vets, Bristol between September 2016 and October 2017. These samples had been sent through the onsite diagnostic laboratory under the direction of the veterinary clinician involved in each individual case, to be used for various diagnostic testing.

Only those cats that had had blood samples taken for a serum biochemistry profile and routine haematology profile, as well as a urine sample for a urinalysis, within a 24-hour period of each other were included, the results for which were all recorded. A minimum of 100µl of surplus whole blood sample was necessary to perform the PCR analysis. If this was not available, the patient was excluded from the study.

The whole blood samples of the patients were stored at 4°C for a period of 7-14 days before DNA extraction commenced. The extracted DNA was stored at 4°C for a maximum of 24 hour prior to PCR analysis.

2.1.2 Categorisation of patients

The cats were initially divided into two main groups: Kidney Disease (KD group) and Non-Kidney Disease (non-KD group). If it was unclear to which group the cat belonged to, it was placed into a ‘query group’, to be further analysed. This was done using biochemistry and urinalysis results and the IRIS staging of CKD in cats (IRIS 2015) as a guideline. This part of the process is displayed in stage 1 of the flowchart in Figure 1. Those with a urine specific gravity (USG) below 1.035 were placed in the KD group. Those with a USG equal or greater than 1.035 were then sorted according to the serum creatinine values. Those with a serum creatinine value <140mmol/l were placed into the non-KD group and those equal to, or above, that value were placed into a query group to be further analysed.

Stage 2 of categorisation, as shown in Figure 1, was then used to organise the cats into groups of chronic kidney disease (CKD group) and non-chronic kidney disease (non-CKD group). The patient history, clinician case reports and further diagnostics results of the new groups were then examined and those which had one or more of the exclusion criteria (Section 2.1.3) were removed from the analysis. Due to the diagnosis of acute kidney injury being an exclusion factor, any cats remaining in the KD group were then able to be placed into the CKD group, as an acute condition has been ruled out. Any cats remaining in the non-KD group were then placed into the non-CKD group.

After the exclusion phase, the clinical history of any cats that remained in the query group were then analysed. Those with no obvious kidney impairment noted, and therefore no obvious renal cause of the elevated creatinine levels,

were placed into the Non-CKD group, and those with possible renal cause were placed into the CKD group. There were 5 cats in which the appropriate group was not obvious from the clinical records; these were then examined by an experienced clinician (Christina Maunder BVM&S CertSAM DipECVIM-CA MRCVS), who allotted them groups.

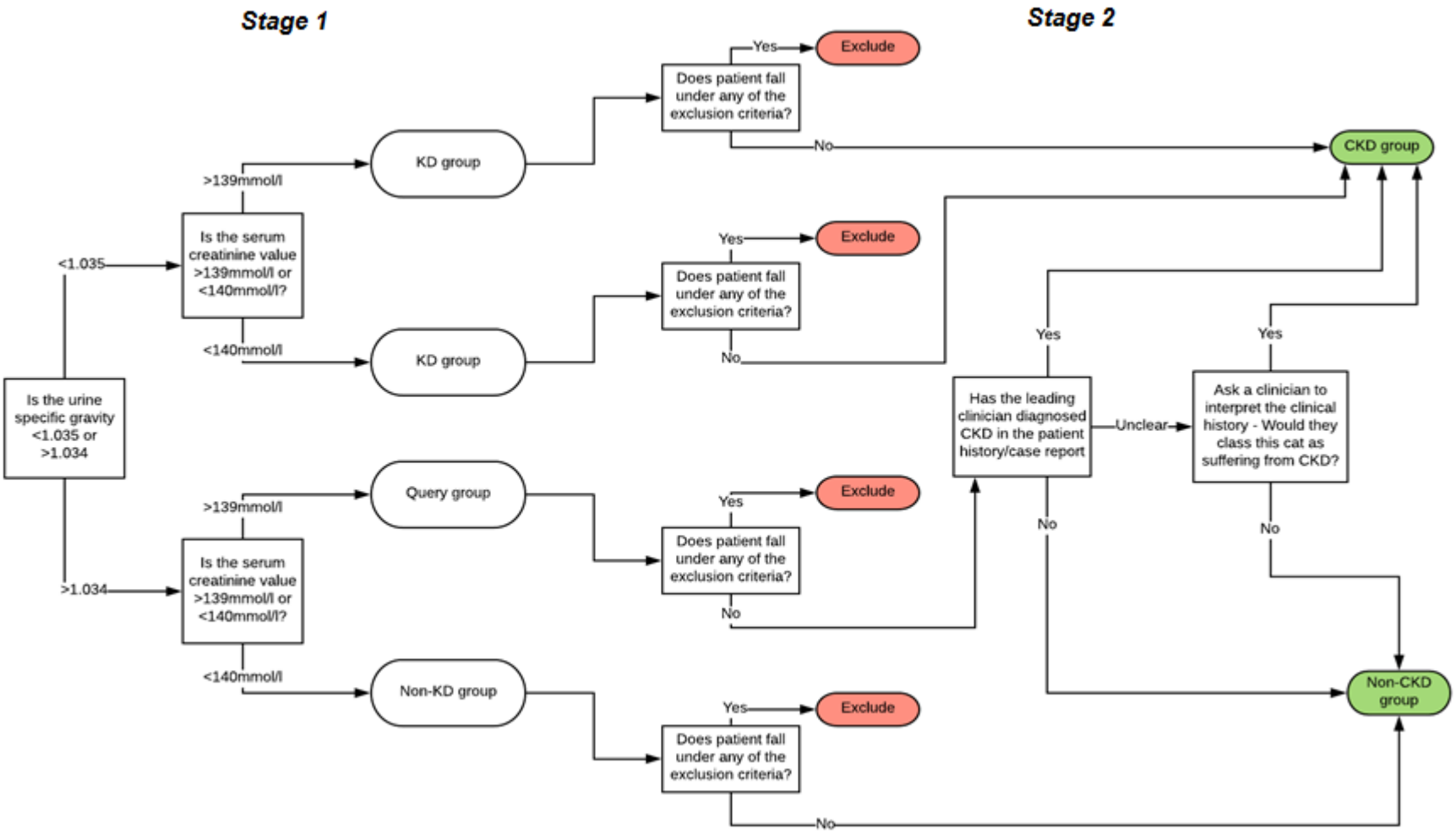

Figure 1 Flowchart of the process of categorisation of cats into the CKD group or Non-CKD group

2.1.3 Exclusion criteria

When analysing the correlation between CKD and leptospiral positivity, certain factors that could be a primary cause of, or predisposing factor for, CKD, were reason for exclusion of those patients from this part of the study (Section 1.1.3), as were conditions that may lead to the misdiagnosis of CKD. This included the following:

- Persian, Oriental, Himalayan, Exotic, Abyssinian or Somalian breeds (Lees 1996, Greco 2001, Beck and Lavelle 2001, Rodriguez et al. 2014, Boyce et al. 1984)

- Cats with reported ingestion of nephrotoxic substances such as lilies or ethylene glycol in the 3 months prior to the blood sample being taken (Fitzgerald 2010, Balakrishnan and Drobatz 2013, Reynolds and Lefebvre 2013)

- Cats with confirmation of FIV-positivity during the investigations performed at Langford Veterinary Services during visit (Poli et al. 1995)

- Reported evidence of previous or current urolithiasis/nephrolithiasis in the clinical notes or diagnostic imaging results (Reynolds and Lefebvre 2013, Kyles et al. 2005)

- Reported evidence of renal neoplasia in the clinical notes or diagnostic imaging results (Reynolds and Lefebvre 2013, Gabor et al. 1998, Gabor et al. 2000)

- Cats diagnosed with current acute kidney injury (AKI) stated in the case reports, discharge instructions or clinical notes to ensure cats included were suffering from chronic renal damage

- Cats classified as biochemically hyperthyroid with a thyroxine value >60nmol/l (Wakeling et al. 2008, Adams et al. 1997a, Becker et al. 2000, Williams et al. 2010, McLoughlin et al. 1993)

Due to inconsistent history recording amongst the clinical notes, identifying cats that had received potentially nephrotoxic medications such as tetracycline, cyclophosphamide or non-steroidal anti-inflammatory drugs within the last 3 months (Papich 2005, Clark 1977, Sparkes et al. 2010, Sykes et al. 2011), proved difficult and therefore this was not used as an exclusion factor.

When looking at the correlations between other variables and leptospiral positivity, all cats tested were included.

2.1.4 Data collection

In addition to the data collected for categorisation and exclusion of samples, the haematology, biochemistry and urinalysis results were recorded, as well as any virology or leptospiral serology results, if performed. Demographic information such as sex, age, neuter status, vaccination status, presence of other pets in the household and outdoor access was also collected and entered into a database for analysis.

2.2 Procedures

2.2.1 Sample collection

Routine haematology and biochemistry profile analyses were performed on the whole blood sample (collected in a tube containing the anticoagulant, Ethylenediaminetetraacetic acid) and the serum sample of each cat, respectively, as well as a urinalysis of each cats' urine sample. These tests were performed independently of this study. Cats were only included in the study if these were all performed within 24 hours of each other. The results of the tests, performed by the onsite diagnostics laboratory, were then used to confirm the absence or presence of CKD (Section 2.1.2, Figure 1), whilst only the whole blood sample was analysed using the PCR assay.

2.2.2 DNA extraction

2.2.2.1 Patient samples

DNA was extracted from the samples using the DNeasy® Blood and Tissue Kit (QIAGEN; #69506). This was done so according to the 'Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol)' in the DNeasy Blood and Tissue Handbook (https://www.qiagen.com/gb/resources/resourcedetail?id=6b09dfb8-6319-464d-996c-79e8c7045a50&lang=en). 100µl of each whole blood sample was used for DNA extraction. The steps for nonnucleated erythrocytes were followed and 100µl of AE buffer used for the final elution process.

2.2.2.2 Positive and negative control

DNA was extracted from a Nobivac® L4 vaccine (MSD; Lot A049A02; Exp 0-2019) using the same protocol as that used for the patient samples (Section 2.2.2). The active substances in this vaccination includes three inactivated Leptospira strains of serovars representing different serogroups within *Leptospira interrogans* species, as well as one strain of a serovar representing a serogroup within the *Leptospira kirschneri* species (Appendix 1). This was diluted to 1:10 with DNase and RNase free water. The negative control used for this study was DNase and RNase free water. Both controls were included in each Real-Time Polymerase Chain Reaction analysis performed.

2.2.3 Real-Time Polymerase Chain Reaction

2.2.3.1 Primers

The following oligonucleotide primers and probe were designed by Dr Tristan Cogan at the University of Bristol and validated during preliminary tests (Section 3.1) with successful amplification of a section of the *lipL32* gene, found in pathogenic species of *Leptospira* (Section 1.3.5.1). The primers and probe were used at a concentration of 10pmol/µl and 2 pmol/µl, and diluted 1:10 and 1:50 with DNase and RNase free water, respectively.

Forward Primer:	E284f 5’ TTC GTA TGA TTT CCC CAA CC 3’
Reverse Primer:	F407r 5’ CGG ATC CAA GTA TCA AAC CAA 3’
Probe:	FAM 5’ AGG CGA CGC TTT CAA AGC GG 3’ TAMRA

2.2.3.2 PCR assay

The quantitative PCR analysis targeting the *lipL32* gene was performed using the Stratagene Mx3005P qPCR System (Agilent Technologies) and the application of the 'Quantitative PCR (Multiple Standards)' experiment type with MxPro QPCR Software (Agilent Technologies; #41454) for the display and analysis of data. The reference dye utilised was FAM.

A total reaction volume of 25µl made up of 12.5µl of 2X Brilliant III QPCR Master Mix (Agilent Technologies; #600880.5), 1µl of each oligonucleotide primer dilution, 0.5 µl of the probe dilution, and 10µl of extracted DNA sample, were used to amplify the target sequence. The negative and positive control were analysed with each test run.

The optimal cycling conditions demonstrated by preliminary testing and used for all PCR reactions in this study commenced with a denaturation step at 95°C (10 minutes) followed by 50 cycles of a further denaturation at 95°C (10 seconds) and an annealing step at 58°C (25 seconds).

MxPro QPCR Software (Agilent Technologies; #41454) was used to display the results of the real-time PCR assay. Samples that tested negative for the presence of pathogenic *Leptospira* were displayed by a complete absence of Threshold Cycle Number (Ct). Any samples with a positive Ct value were deemed positive for *Leptospira*.

2.2.4 DNA sequencing

2.2.4.1 PCR product purification

To verify PCR product identity, The QIAquick® Purification Kit (QIAGEN; #28104) was used according to the manufacturer's instructions to purify the PCR products prior to DNA sequencing. 10µl of the PCR product was mixed with 50µl of Buffer PB as a starting volume. After processing the samples, 15µl of the eluted volumes were then premixed with 2µl of a 1/10 dilution of the forward primer (Section 2.2.3.1).

The premixed samples were sent to the Eurofins Genomics Laboratory in Germany for sequencing.

A small number of the sequences were aligned using CLC Sequence Viewer 7 and verified as *Leptospira*.

2.2.5 Statistical Analysis

Statistical analyses were performed using GraphPad QuickCalcs software (https://www.graphpad.com/quickcalcs/contingency1) and IBM SPSS Statistics 24 software, when investigating the association between all the identified variables against leptospiral positivity. Two types of analysis were used: A two-tailed Fisher's exact test and Binary logistic regression analysis.

When analysing the association between cats with CKD and leptospiral positivity, those that belonged to one of the exclusion criterion (Section 2.1.3), were removed and the two-tailed Fisher's exact test analysis was performed on the two sets of data (Section 3.3).

In order to explore the relationship between the different variables of *all* cats and leptospiral positivity (Section 3.4), both types of analyses were used depending on the data type. The two-tailed Fisher's exact test was used when dealing with variables of binary value and the binary logistic regression was used when dealing with variables of a scale value such as blood and urine test results, and age.

3 Results

3.1 Preliminary Data

3.1.1.1 Sensitivity Panel

The primers used in the PCR assay (Section 2.2.3) were tested against 15 other bacterium known to cause infection in small animals, as well as the negative and positive controls (Section 2.2.2.2). The only one that produced a positive result for the presence of pathogenic *Leptospira* was the positive control with a Ct value of 34.49, as demonstrated in Table 3-1.

Table 3-1: Sensitivity panel results from the PCR assay used in the current study on the extracted DNA of 15 different species of bacterium

Bacteria species	Ct number (dR)
Staphylococcus aureus	No Ct
Staphylococcus intermedius	No Ct
Escherichia coli	No Ct
Pseudomonas aeruginosa	No Ct
Pasteurella multocida	No Ct
Staphylococcus epidermidis	No Ct
Enterococcus faecalis	No Ct
Erysipelothrix rhusiopathiae	No Ct
Streptococcus pyogenes	No Ct
Salmonella enterica	No Ct
Proteus vulgaris	No Ct
Bacillus cereus	No Ct
Mycobacterium bovis	No Ct
Clostridium glycolicum	No Ct
Bacteroides fragilis	No Ct
Leptospira	34.49
Water	No Ct

3.1.1.2 Efficiency Curve

Using DNA extracted from the L4 vaccine as a template, it was found that between levels of 10-1000 copies, the qPCR assay used in the current study has an efficiency of 89.1%, with an R^2 of 0.9777, as shown on the efficiency curve displayed in Figure 2.

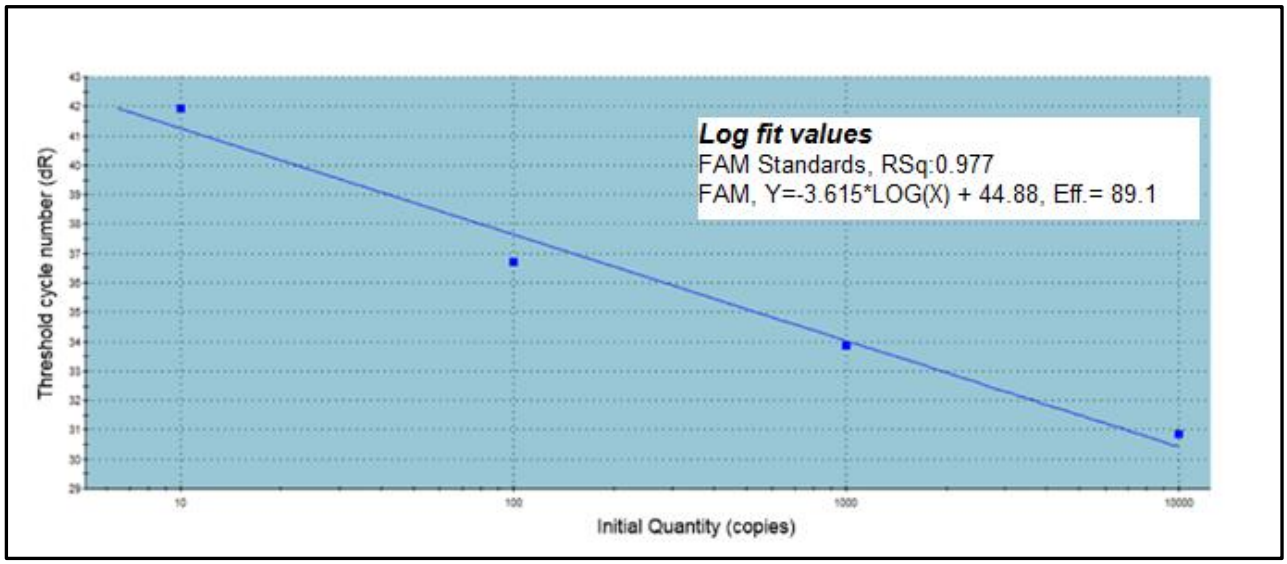

Figure 2 Efficiency curve of leptospiral qPCR assay

3.2 Threshold cycle number (Ct) of leptospiral positive samples

In total, 24/158 cats (15.19%) cats were found to be leptospiral positive by qPCR of the whole blood sample, the Ct values of which are shown Table 3-2. Any value above zero represented successful amplification of the target sequence and the sample was considered leptospiral positive. In order to investigate the relationship between CKD and leptospiral positivity (Section 3.3), the cats were categorised as ‘CKD’ or ‘non-CKD’, and those that fitted certain criteria were excluded from this analysis (Section 2.1.3). To study the relationship between other variables such as demographic information and blood and urine analysis results (Section 3.4), any of the previously excluded samples were then incorporated for these statistical analyses. The original allocated groups of the leptospiral cats are also displayed in Table 3-2.

Table 3-2: The threshold cycle number (Ct) of all the leptospiral positive cats and their allocated grouping

Sample number	Ct number (dR)	Original grouping
1	39.38	CKD
2	39.20	Non-CKD
3	38.70	Non-CKD
4	38.48	CKD
5	38.42	Excluded
6	38.71	Excluded
7	38.15	Non-CKD
8	39.39	Excluded
9	38.35	Excluded
10	37.75	Excluded
11	38.10	Excluded
12	38.24	Non-CKD
13	38.75	Excluded
14	39.43	Excluded
15	37.89	Non-CKD
16	38.46	Non-CKD
17	38.15	Excluded
18	37.54	Excluded
19	38.44	Excluded
20	38.03	Excluded
21	40.07	Excluded
23	39.83	Excluded
24	38.15	Excluded

3.3 Investigating the association between leptospiral positivity and chronic kidney disease in cats

In total, 158 cats were recruited for this study. In order to investigate the correlation between leptospiral positivity and chronic kidney disease, 69 cats were excluded (Section 2.1.3). Reasons for this included the following: cats belonging to breeds predisposed to congenital kidney abnormalities (n=4), nephropathy of toxic origin within the last 28 days (n=1), reported evidence of renal neoplasia during sampling visit (n=1), reported evidence of current (n=18) or previous (n=14) (or both (n=7)) urolithiasis or nephrolithiasis, clinician has made a diagnosis of AKI in the final case report (n=8) and finally, those cats who were biochemically hyperthyroid (thyroxine>60nmol/l) (n=37). Some cats fitted more than one exclusion criterion.

Of the remaining 89 cats, 56 (62.92%) belonged to the non-CKD group and a total of 33 (37.08%) belonged to the CKD group. The CKD group consisted of 20 (60.61%) females and 13 (39.39%) males of ages ranging between 1-19 years (Mean age = 11.55 years). The non-CKD group consisted of 30 (53.57%) females and 26 (46.43%) males aged between 4 months and 17 years of age (mean age = 9.13). Table 3-3 demonstrates the number of cats with a leptospiral positive PCR result within these groups.

Table 3-3: Percentage of leptospiral positive cats within the different groups

Group	Leptospiral positive	Leptospiral negative	Total (n)
CKD group	2 (6.06%)	31 (93.94%)	33
Non-CKD group	6 (10.71%)	50 (89.29%)	56
Total (n)	8 (8.99%)	81 (91.01%)	89

A two-tailed Fisher's exact test analysis was performed on the values shown in Table 3-3. The two-tailed P value was equal to 0.7049 and considered not be statistically significant.

3.4 Investigating the association between all other variables and leptospiral positivity

3.4.1 Demographic variables

The demographic variables are demonstrated in Table 3-4 and Table 3-5, including age, sex, neuter and vaccination status, presence of other animals in the household, access to outdoors and the breed. The total number of leptospiral positive cats within each variable group is also displayed.

Binary logistic regression analysis was used to investigate the association between age and leptospiral positivity, as shown in Table 3-6. Leptospiral positivity was found to be significantly negatively associated with age, with a P value of 0.016.

The two-tailed Fisher's exact test was used to analyse the relationship between the demographic variables and leptospiral positivity, as shown in Table 3-7. The cats for which the information was not available were excluded prior to each statistical analysis. The same statistical analysis was performed to investigate the relationship between breed and leptospiral positivity, shown in Table 3-8. Nothing was found to be statistically significant within these sets of variables.

Table 3-4: Demographic information of all cats

Variable	Total	% of total (n=158)	Leptospiral positive (% of variable total)
Age			
≤ 5 years	33	20.89	10 (30.3%)
6-10 years	42	26.58	4 (9.52%)
> 10 years	83	52.53	10 (12.05%)
Sex			
Female	88	55.70	10 (11.36%)
Male	70	44.30	14 (20%)
Neuter status			
Neutered	154	97.47	23 (14.94%)
Entire	4	2.53	1 (25%)
Vaccination status			
Up to date	71	44.94	16 (22.54%)
Out of date/Never	14	8.86	1 (7.14%)
Unknown	73	46.20	7 (9.59%)
Other pets in household			
Dog	15	9.49	5 (33.33%)
Cat	57	36.08	12 (21.05%)
Other	1	0.63	0 (0%)
None	7	4.43	0 (0%)
Unknown	88	55.69	10 (11.36%)
Outdoor access			
Yes	69	43.67	14 (20.29)
No	10	6.33	1 (10%)
Unknown	79	50.00	9 (11.39%)

Table 3-5: Total of cats belonging to each breed

Variable	Total	% of total (n=158)	Leptospiral positive (% of variable total)
Breed			
Domestic Short Hair	118	74.68	15 (12.71%)
Domestic Long Hair	15	9.49	5 (33.33%)
British Domestic Short Hair	2	1.27	1 (50%)
Siamese	2	1.27	1 (50%)
Maine coon	6	3.8	1 (16.67%)
Birman	1	0.63	0 (0%)
Ragdoll	3	1.9	0 (0%)
Bengal	3	1.9	0 (0%)
Burmese	2	1.27	0 (0%)
*Somali**	1	0.63	1 (100%)
*Persian**	1	0.63	0 (0%)
*Exotic**	0	0	-
*Himalayan**	0	0	-
*Oriental**	0	0	-
*Abyssinian**	2	1.27	0 (0%)
Crossbreed	2	1.27	0 (0%)

*Breeds predisposed to congenital renal abnormalities that could lead to CKD (Section 1.1.3.1)

Table 3-6: Binary logistic regression analysis of age vs leptospiral positivity of all cats

Variable	Degree of Freedom (df)	Significance (Sig.)	(B)	Odds Ratio
Age	1	0.016	-1.1720	0.179

Table 3-7: Two-tailed Fisher's exact test analysis of demographic variables vs leptospiral positivity of all cats

Variable	Leptospiral positive by PCR	Leptospiral negative by PCR	Total	Two-tailed P value
Sex				
Female	10	78	88	
Male	14	56	70	**0.1804**
Total	124	134	158	
Neuter status				
Neutered	23	131	154	
Entire	1	3	4	**0.4862**
Total	24	134	158	
Vaccination status				
Up to date	16	55	71	
Out of date/Never	1	13	14	**0.2829**
Total	17	68	85	
Dogs in household				
Yes	5	10	15	
No	9	46	55	**0.1607**
Total	14	56	70	
Another cat in household				
Yes	12	45	57	
No	2	11	13	**1.000**
Total	14	56	70	
'Other' pets in household				
Yes	0	1	1	
No	14	55	69	**1.000**
Total	14	56	70	
Outdoor Access				
Yes	14	55	69	
No	1	9	10	**0.6774**
Total	15	64	79	

Table 3-8: Two-tailed Fisher's exact test analysis of breed vs leptospiral positivity of all cats

Variable	Leptospiral positive by PCR	Leptospiral negative by PCR	Total	Two-tailed P value
Domestic Short Hair				
Yes	15	103	118	
No	9	31	40	**0.2002**
Total	24	134	158	
Domestic Long Hair				
Yes	5	10	15	
No	19	124	143	**0.0551**
Total	24	134	158	
British Domestic Short Hair				
Yes	1	1	2	
No	23	133	156	**0.2815**
Total	24	134	158	
Siamese				
Yes	1	1	2	
No	23	133	156	**0.2815**
Total	24	134	158	
Maine coon				
Yes	1	5	6	
No	23	129	152	**1.000**
Total	24	134	158	
Birman				
Yes	0	1	1	
No	24	133	157	**1.000**
Total	24	134	158	
Ragdoll				
Yes	0	3	3	
No	24	131	155	**1.000**
Total	24	134	158	
Bengal				
Yes	0	3	3	
No	24	131	155	**1.000**
Total	24	134	158	
Burmese				
Yes	0	2	2	
No	24	132	156	**1.000**
Total	24	134	158	
Somali				
Yes	1	0	1	
No	23	134	157	**0.1519**
Total	24	134	158	
Persian				
Yes	0	1	1	
No	24	133	157	**1.000**
Total	24	134	158	
Abyssinian				
Yes	0	2	2	
No	24	132	156	**1.000**
Total	24	134	158	
Crossbreed				
Yes	0	2	2	
No	24	132	156	**1.000**
Total	24	134	158	

3.4.2 Haematological variables

Table 3-9 shows the haematology results for all the cats recruited for the study, grouped into those found to be leptospiral positive (n=24) and leptospiral negative (n=134), by PCR. This included the units and the reference intervals used by Langford Vets Diagnostics Laboratories as well as the range and mean values between the two groups. Also shown, are the number of cats for which the individual values were not recorded; these cats were excluded prior to statistical analysis.

Binary logistic regression analysis was used to investigate the relationship between the haematological values and leptospiral positivity, as shown in Table 3-10. The values of statistical significance were lymphocyte and basophil count, both having a positive correlation to leptospiral positivity by PCR with P values of 0.029 and 0.032, respectively.

Table 3-11 shows the results of the two-tailed Fisher's exact test analysis used to investigate the association between haematological values outside of the laboratory reference interval (RI) and leptospiral positivity. The variables with significant association to leptospiral positivity were lymphocytes and eosinophil counts above the RI (lymphocytosis and eosinophilia), with two-tailed P values of 0.0223 and 0.0203, respectively. Monocyte and basophil count below the RI were not included in the analysis as the lowest RI range was zero.

Table 3-9: Haematological values of all cats

Variable	Units	Reference Interval	PCR Positive cats (n=24)			PCR Negative cats (n=134)		
			Range	Mean value	Not recorded	Range	Mean value	Not Recorded
Haematology Results								
Haemoglobin	g/dL	8.10-14.20	4.2-13.7	10.34	0	3.4-15.6	10.97	1
Red Blood cell	x10^12/l	6-10.10	3.1-9.21	7.18	0	2.82-12.33	7.72	0
Haematocrit	%	27.7-46.8	11.9-41.3	31.07	0	11.2-47.1	33.1	0
White Blood Cell	x 10^9/l	6.30-19.60	5.11-40.28	13.18	0	3.08-46.66	11.68	0
Platelets	x 10^9/l	156-626	67-484	272.5	0	18-749	244.15	0
Mean Cell Volume	fL	41.3-52.6	38.6-49.6	43.74	0	28.4-60.2	43.02	0
Mean Cell Haemoglobin	pg	12-16	12.7-16.6	14.5	0	11.5-18.1	14.24	1
Mean Cell Haemoglobin Concentration	g/dL	27-32.8	31.7-35	33.34	0	30-36.8	33.09	1
Neutrophils	x 10^9/l	3-13.4	2.08-26.81	8.48	0	1.73-41.54	8.38	0
Lymphocytes	x 10^9/l	2-7.2	0.79-10.07	3.08	0	0.14-7.15	2.28	0
Monocytes	x 10^9/l	0-1	0.08-1.21	0.4	0	0.03-2.62	0.34	0
Eosinophils	x 10^9/l	0.3-1.7	0-11.68	1.21	0	0-10.32	0.69	0
Basophils	x 10^9/l	0-0.1	0-1.61	0.09	0	0-0.82	0.02	1

Table 3-10: Binary logistic regression analysis of haematological values vs leptospiral positivity of all cats

Variable	Degree of Freedom (df)	Significance (Sig.)	(B)	Odds Ratio
Haemoglobin	1	0.201	-0.518	0.596
Red Blood Cells	1	0.116	0.129	1.137
Haematocrit	1	0.145	0.092	1.096
White Blood cells	1	0.372	-0.156	0.856
Platelets	1	0.454	0.001	1.001
Mean Cell Volume	1	0.528	-0.015	0.985
Mean Cell Haemoglobin	1	0.284	0.276	1.318
Mean Cell Haemoglobin Concentration	1	0.360	0.301	1.351
Neutrophils	1	0.970	0.130	1.139
Lymphocytes	1	0.029	0.351	1.421
Monocytes	1	0.426	0.021	1.021
Eosinophils	1	0.092	0.100	1.105
Basophils	1	0.032	1.531	4.624

Table 3-11: Two-tailed Fisher's exact test analysis of haematological values outside of the reference interval (RI) vs leptospiral positivity of all cats

Variable	Leptospiral positive by PCR	Leptospiral negative by PCR	Total	Two-tailed P value
Haemoglobin > RI				
Yes	0	7	7	
No	24	126	150	**0.5960**
Total	24	133	157	
Haemoglobin < RI				
Yes	5	14	19	
No	19	119	138	**0.1742**
Total	24	133	157	
Red Blood Cells > RI				
Yes	0	4	4	
No	24	130	154	**1.000**
Total	24	134	158	
Red Blood Cells < RI				
Yes	5	19	24	
No	19	115	134	**0.3702**
Total	24	134	158	
Haematocrit > RI				
Yes	0	1	1	
No	24	133	157	**1.000**
Total	24	134	158	
Haematocrit < RI				
Yes	5	21	26	
No	19	113	132	**0.5524**
Total	24	134	158	
White Blood Cell > RI				
Yes	5	12	17	
No	19	122	141	**0.1423**
Total	24	134	158	
White Blood Cell < RI				
Yes	4	22	26	
No	20	112	132	**1.000**
Total	24	134	158	
Platelets > RI				
Yes	0	2	2	
No	24	132	156	**1.000**
Total	24	134	158	
Platelets < RI				
Yes	6	53	59	
No	18	81	99	**0.2518**
Total	24	134	158	
Mean Cell Volume > RI				
Yes	0	3	3	
No	24	131	155	**1.000**
Total	24	134	158	
Mean Cell Volume < RI				
Yes	8	36	44	
No	16	98	114	**0.6212**
Total	24	134	158	

Table 3-11 continued

Variable	Leptospiral positive by PCR	Leptospiral negative by PCR	Total	Two-tailed P value
Mean Cell Haemoglobin > RI				
Yes	2	8	10	
No	22	125	147	**0.6510**
Total	24	133	157	
Mean Cell Haemoglobin < RI				
Yes	0	6	6	
No	24	127	151	**0.5915**
Total	24	133	157	
Mean Cell Haemoglobin Concentration > RI				
Yes	18	80	98	
No	6	53	59	**0.2517**
Total	24	133	157	
Mean Cell Haemoglobin Concentration < RI				
Yes	0	0	0	
No	24	133	157	**1.000**
Total	24	133	157	
Neutrophils > RI				
Yes	5	19	24	
No	19	115	134	**0.3702**
Total	24	134	158	
Neutrophils < RI				
Yes	2	5	7	
No	22	129	151	**0.2879**
Total	24	134	158	
Lymphocytes > RI				
Yes	2	0	2	
No	22	134	156	**0.0223**
Total	24	134	158	
Lymphocytes < RI				
Yes	9	70	79	
No	15	64	79	**0.2676**
Total	24	134	158	
Monocytes > RI				
Yes	2	5	7	
No	22	129	151	**0.2879**
Total	24	134	158	
Eosinophils > RI				
Yes	5	7	12	
No	19	127	146	**0.0203**
Total	24	134	158	
Eosinophils < RI				
Yes	8	53	61	
No	16	81	97	**0.6527**
Total	24	134	158	
Basophils > RI				
Yes	3	5	8	
No	21	129	150	**0.1032**
Total	24	134	158	

3.4.3 Biochemical variables

Table 3-12 summarises the biochemical values of all the cats recruited for the study. This included the range, mean values including the units used, as well as the reference intervals used by Langford Vets Diagnostics Laboratories, where the tests were carried out on the serum samples. These figures are displayed for the leptospiral positive cats (n=24) and leptospiral negative cats (n=134), respectively. Also shown are the number of cats for which the individual values were not recorded, these cats were excluded prior to statistical analysis.

As with the haematological values, a binary logistic regression analysis was implemented to investigate the relationship between the biochemical values and leptospiral positivity, shown in Table 3-13. The only value showing a significant association was alanine amino transferase, with a P value of 0.022, showing a positive correlation to leptospiral positivity. Phosphorus/phosphate, cholesterol and thyroxine levels were not included in this analysis due to insufficient number of cats for which these values were recorded.

A two-tailed Fisher's exact test was also performed on these values to investigate the relationship between biochemical values above or below the reference interval (RI) and leptospiral positivity, as shown in Table 3-14. Values of significance were, a total protein value below the RI with a two-tailed P value of 0.0290 and a total thyroxine value below the RI with a two-tailed P value of 0.0348, showing a negative association with leptospiral positivity. A significant positive correlation was also found between thyroxine value above the RI and leptospiral positivity with a two-tailed P value of 0.0279.

Table 3-12: Biochemical values of all cats

Biochemical values	Units	Reference Interval	PCR Positive cats (n=24)			PCR Negative cats (n=134)		
			Range	Mean value	Not recorded	Range	Mean value	Not Recorded
Biochemical values								
Serum Creatinine	µmol/l	133-175	23-1645	213.04	0	27-871	136.3	0
Urea	mmol/l	6.5-10.5	3.4-97.9	14.5	0	3.7-50	11.81	0
Total Protein	g/l	77-99	43.6-97.1	68.14	1	51-104.6	65.49	3
Albumin	g/l	24-35	14.6-33.5	27.1	2	18.9-32.9	27.22	3
Globulin	(g/l	21-51	29-75.1	41	2	23.1-85.2	38.27	3
A:G Ratio		0.4-1.3	0.29-0.85	0.68	2	0.23-1.21	0.73	3
Alanine aminotransferase	U/l	15-45	21-1845	181.26	1	22-729	105.73	3
Alkaline Phosphatase	U/l	15-60	4-483	65.7	1	1-309	50.7	3
Sodium	mmol/l	149-157	147.2-159.6	154.5	9	124-168.8	153.83	31
Potassium	mmol/l	4-5	3.61-4.76	4.25	8	2.74-5.6	4.26	31
Chloride	mmol/l	115-130	116-123	119.67	9	89-124	117.69	34
Calcium	mmol/l	2.3-2.5	1.74-2.82	2.45	1	1.77-3.1	2.47	14
*Phosphorus/Phosphate***	mmol/l	0.95-1.55	1.03-3.88	1.67	3	0.81-4.4	1.62	8
*Cholesterol***	Mmol/l	3-6.9	2-9	4.44	15	2-7	3.9	116
Thyroxine **	nmol/l	15-60	25.9-393	160.13	11	12-749	84.17	49

*A laboratory value of '<12.5' was recorded as 12 for final analysis

** These results were not included in the binary logistic regression analysis due to insufficient numbers of cats for which they were measured

Table 3-13: Binary logistic regression analysis of biochemical variables vs leptospiral positivity

Variable	Degree of Freedom (df)	Significance (Sig.)	(B)	Odds Ratio
Serum Creatinine	1	0.073	-0.013	0.987
Urea	1	0.246	-0.062	0.940
Total Protein	1	0.287	-0.045	0.956
Albumin	1	0.664	0.367	1.444
Globulin	1	0.339	-8.569	0.000
Albumin to globulin ratio	1	0.273	0.002	1.002
Alanine aminotransferase	1	0.022	0.003	1.003
Alkaline phosphatase	1	0.102	-0.082	0.921
Sodium	1	0.584	0.042	1.043
Potassium	1	0.809	0.384	1.468
Chloride	1	0.072	1.266	3.545
Calcium	1	0.652	-0.013	0.000

Table 3-14: Two-tailed Fisher's exact test analysis of biochemical values outside of the reference interval (RI) vs leptospiral positivity of all cats

Variable	Leptospiral positive by PCR	Leptospiral negative by PCR	Total	Two-tailed P value
Serum Creatinine > RI				
Yes	4	26	30	
No	20	108	128	**1.000**
Total	24	134	158	
Serum Creatinine < RI				
Yes	18	86	104	
No	6	48	54	**0.3572**
Total	24	134	158	
Urea > RI				
Yes	9	59	68	
No	15	75	90	**0.6566**
Total	24	134	158	
Urea < RI				
Yes	5	16	21	
No	19	118	137	**0.3225**
Total	24	134	158	
Total Protein > RI				
Yes	1	1	2	
No	22	130	152	**0.2772**
Total	23	131	154	
Total Protein < RI				
Yes	19	126	145	
No	4	5	9	**0.0290**
Total	23	131	154	
Albumin > RI				
Yes	0	0	0	
No	22	131	153	**1.000**
Total	22	131	153	
Albumin < RI				
Yes	2	14	16	
No	20	117	137	**1.000**
Total	22	131	153	
Globulin > RI				
Yes	1	5	6	
No	21	126	147	**1.000**
Total	22	131	152	
Globulin < RI				
Yes	0	0	0	
No	22	131	153	**1.000**
Total	22	131	153	
A:G > RI				
Yes	0	0	0	
No	22	131	153	**1.000**
Total	22	131	153	
A:G < RI				
Yes	1	2	3	
No	21	129	150	**0.3744**
Total	22	131	153	

Table 3-14 continued

Variable	Leptospiral positive by PCR	Leptospiral negative by PCR	Total	Two-tailed P value
Alanine aminotransferase > RI				
Yes	17	98	115	
No	6	33	39	**1.000**
Total	23	131	154	
Alanine aminotransferase < RI				
Yes	0	0	0	
No	23	131	154	**1.000**
Total	23	131	154	
Alkaline phosphatase > RI				
Yes	8	38	46	
No	15	93	108	**0.6240**
Total	23	131	154	
Alkaline phosphatase < RI				
Yes	3	13	16	
No	20	118	138	**0.7099**
Total	23	131	154	
Sodium > RI				
Yes	3	18	21	
No	12	85	97	**0.7294**
Total	15	103	118	
Sodium < RI				
Yes	2	6	8	
No	13	97	110	**0.2687**
Total	15	103	118	
Potassium > RI				
Yes	0	9	9	
No	16	94	110	**0.6067**
Total	16	103	119	
Potassium < RI				
Yes	4	32	36	
No	12	71	83	**0.7738**
Total	16	103	119	
Chloride > RI				
Yes	0	0	0	
No	15	100	115	**1.000**
Total	15	100	115	
Chloride < RI				
Yes	0	9	9	
No	15	91	106	**0.6031**
Total	15	100	115	

Table 3-14 continued

Variable	Leptospiral positive by PCR	Leptospiral negative by PCR	Total	Two-tailed P value
Calcium > RI				
Yes	11	50	61	
No	12	70	82	**0.6484**
Total	23	120	143	
Calcium < RI				
Yes	5	21	26	
No	18	99	117	**0.5699**
Total	23	120	143	
Phosphate/Phosphorus > RI				
Yes	13	58	71	
No	8	68	76	**0.2388**
Total	21	126	147	
Phosphate/Phosphorus < RI				
Yes	0	3	3	
No	21	123	144	**1.000**
Total	21	126	147	
Cholesterol > RI				
Yes	2	2	4	
No	7	16	23	**0.5815**
Total	9	18	27	
Cholesterol < RI				
Yes	1	2	3	
No	8	16	24	**1.000**
Total	9	18	27	
Thyroxine > RI				
Yes	9	28	37	
No	4	57	61	**0.0279**
Total	13	85	98	
Thyroxine < RI				
Yes	0	25	25	
No	13	60	73	**0.0348**
Total	13	85	98	

3.4.4 Urinalysis variables

The results of the urinalysis tests are shown in Table 3-15. The mean value, range and number of cats for which individual values were not recorded are shown for both the leptospiral positive cats (n=24) and leptospiral negative cats (n=134), as well as the units and reference intervals used by the Langford Vets Diagnostic Laboratories, where the tests were performed.

Table 3-16 shows the range and mean urinary pH for cats with and without the presence of uroliths or nephroliths.

A binary logistic regression analysis was performed to investigate the relationship between the urinalysis test results and leptospiral positivity by PCR of all cats recruited for the study. The results are displayed in Table 3-17. None of the test results were found to be statistically significant.

Table 3-15: Urinalysis values for all cats

Urinalysis Results	Units	Reference interval	PCR Positive cats (n=24)			PCR Negative cats (n=134)		
			Range	Mean value	Not recorded	Range	Mean value	Not Recorded
Protein*	mg/dl	≥0	2.4-598.1	62.1	0	1.9-1510.8	54.04	0
Creatinine	mmol/l	≥0	2.4-62.8	11.54	0	2.1-59.3	16.01	0
Protein/Creatinine Ratio*	Ratio	0-0.5	0.04-5.57	0.65	0	0.03-7.3	0.38	0
Glucose **	Scale	0-3	0-3	0.13	1	0-3	0.17	1
Ketones**	Scale	0-3	0-1	0.04	1	0-1	0.03	1
Blood**	Scale	0-3	0-3	0.74	1	0-3	0.61	1
USG***	Ratio	1.000->1.050	1.010-1.060	1.031	0	1.010-1.060	1.034	0
pH	Scale		5.8-7.8	6.75	0	4.5-8.7	6.68	0

*A laboratory value of '<8' was recorded as '7' for final analysis

** A Laboratory value '< x' was recorded as '(x - 0.01)' for final analysis

*** A Laboratory value of 'Trace' was recorded as '0.5' for final analysis

**** A Laboratory value of '>1.050' was recorded as '1.060' for final analysis

Table 3-16: Urinary pH of cats with and without reported evidence of urolithiasis or nephrolithiasis

Variable	Cats with reported evidence or uroliths or nephroliths (n=17)		Cats with no reported evidence or uroliths or nephroliths (n=141)	
	Range	Mean	Range	Mean
Urinary pH	6.1-6.7	6.77	4.5-8.7	6.68

Table 3-17: Binary logistical regression analysis of urinalysis variables vs leptospiral positivity of all cats

Variable	Degree of Freedom (df)	Significance (Sig.)	(B)	Odds Ratio
Protein*	1	0.977	0.000	1.000
Creatinine	1	0.122	0.38	0.951
Protein/Creatinine Ratio	1	0.414	0.317	1.144
Glucose ***	1	0.747	0.458	0.729
Ketones***	1	0.618	0.464	2.437
Blood***	1	0.618	0.406	1.158
USG*****	1	0.376	0.359	1007567.414
pH	1	0.628	0.119	1.147

*A laboratory value of '<8' was recorded as '7' for final analysis

** A Laboratory value '< x' was recorded as '(x - 0.01)' for final analysis

*** A Laboratory value of 'Trace' was recorded as '0.5' for final analysis

**** A Laboratory value of '>1.050' was recorded as '1.060' for final analysis

3.4.5 Virology and serology variables

The virology and serology results are displayed for the leptospiral positive (n=24) and leptospiral negative cats (n=134) in Table 3-18. Also shown are the number of cats belonging to each group for which these values were not measure/recorded, which is true for the majority. A two-tailed Fisher's exact test analysis of these values proved none of them to be statistically significant, as shown in Table 3-19.

Table 3-18: Virology and serology results of all cats

Variable	PCR Positive cats (n=24)		PCR Negative cats (n=134)	
	Total	Not recorded	Total	Not recorded
Virology				
FIV positive	0	22	1	119
FeLV positive	0	22	0	120
Serology				
Leptospiral positive	0	23	0	132

Table 3-19: Two-tailed Fisher's exact test analysis of virology and serology results vs leptospiral positivity of all cats

Variable	Leptospiral positive by PCR	Leptospiral negative by PCR	Total	Two-tailed P value
FIV positive				
Yes	0	1	1	
No	2	14	16	**1.000**
Total	2	15	17	
FeLV positive				
Yes	0	0	0	
No	2	14	16	**1.000**
Total	2	14	16	
Leptospiral positive by MAT				
Yes	0	0	0	
No	1	1	2	**1.000**
Total	1	1	2	

3.4.6 Other variables related to CKD

Information gathered from the clinical histories of the cats for the categorisation process (Section 2.1.2) in order to investigate the relationship between CKD and leptospiral positivity are displayed for *all* cats in Table 3-20. The totals for each variable are shown for the leptospiral positive cats (n=24) and leptospiral negative cats (n=134), respectively. A two-tailed Fishers exact test was performed on each variable against leptospiral positivity by PCR, the results of which are also displayed in Table 3-20. The variables of statistical significance were presence of AKI (two-tailed P value = 0.0192) and the presence of current uroliths or nephroliths (two-tailed P value = 0.0344).

Table 3-20: Two-tailed Fisher's exact test analysis of other variables vs leptospiral positivity of all cats

Variable	Leptospiral positive by PCR	Leptospiral negative by PCR	Total	Two-tailed P value
Acute Kidney Injury				
Yes	4	4	8	
No	20	130	150	**0.0192**
Total	24	134	158	
Reported digestion of nephrotoxic substance				
Yes	1	0	1	
No	23	134	157	**0.1519**
Total	24	134	158	
Current uroliths/nephroliths				
Yes	6	12	18	
No	18	122	140	**0.0344**
Total	24	134	158	
History of uroliths/nephroliths				
Yes	2	12	14	
No	22	122	144	**1.000**
Total	24	134	158	

3.4.7 Statistical Models

Table 3-21 shows the results of a binary logistic regression analysis between age and presence of AKI. There was significant negative association with a P value of 0.002.

Table 3-21: Binary logistic regression analysis of AKI vs age of all cats

Variable	Degree of Freedom (df)	Significance (Sig.)	(B)	Odds Ratio
Age vs AKI	1	0.002	-0.186	0.830

4 Discussion

4.1 Investigating the association between CKD and leptospiral positivity

The main aim of the current study was to investigate the presence of leptospiral infection in cats with chronic kidney disease. Although acute pathogenic leptospirosis has rarely been reported amongst cats, evidence of urinary shedding has been found (Rodriguez et al. 2012, Kim et al. 2008, Ferris and Andrews 1965, Larsson et al. 1985), as well as varying levels of seropositivity by MAT worldwide (Obrenovic et al. 2014). It was hypothesised that the kidney damage elicited by leptospiral infection commonly seen in dogs and humans, may also occur in cats, and this bacterial infection may be one of the many possible primary causes of CKD in cats.

After excluding all the cats that showed evidence of other possible aetiology of CKD (Section 2.1.3), there was found to be no significant correlation between CKD and a positive PCR result for *Leptospira* in the blood (Two-tailed P value = 0.7049), when performing the two-tailed Fisher's exact test analysis on the remaining sample number (Section 3.3). In fact, when analysing the data of *all* the cats included in this study, there was a significant positive correlation between leptospiral infection and acute kidney injury (AKI) with a two-tailed P value of 0.0192 (Section 3.4.6, Table 3-20). Rodriguez et al. found that cats with AKI had a higher percentage of seropositivity than those with CKD or those considered healthy, although the significance of this was not reported, and it is unclear as to what a positive MAT result means in terms of active infection (Rodriguez et al. 2014).

Although infrequent, the reported cases of acute leptospiral infection in cats (Arbour et al. 2012, Mason et al. 1972, Bryson and Ellis 1976, Beaudu-Lange and Lange 2014, Rees 1964, Harkness et al. 1970, Reilly et al. 1994) commonly demonstrated signs of AKI (Mugford et al. 2013). Clinical signs reported amongst these cats included, anorexia, lethargy, polyuria/polydipsia, haematuria and vomiting. Further investigations in some cases revealed azotaemia, abnormal kidneys on x-ray, interstitial nephritis and other indicators of AKI.

The presence of *L*. Pomona using diagnostic tools was commonly found amongst these acute cases. For example, two of the three leptospiral positive cats described by Arbour et al had sudden onset of PUPD, as well as other kidney disease related clinical signs (Arbour et al. 2012). Both demonstrated abnormal renal values on serum biochemistry and USG, and abnormally sized kidneys on diagnostic imaging. After receiving antimicrobial therapy, all renal related clinical signs resolved, and kidney values on blood analysis returned to normal. The third cat of this report, had been developing clinical renal signs for several months (by which point, the kidney damage had become chronic) before rapidly deteriorating and euthanasia was performed. All cats tested positive for *L*. Pomona by MAT. Rees reported a cat that had presented to the author for treatment of interstitial nephritis which responded poorly to antibiotics. The cat soon developed jaundice and was also euthanased (Rees 1964) and MAT of the heart blood revealed seropositivity to *L*. Pomona*.* It could be pathogenesis in cats is serovar dependant (Hartmann et al. 2013). However, when experimentally infecting three cats with *L*. Pomona*,* Ferris et al. reported, that in spite of urinary shedding of the bacteria for over a month in one cat, it was not detected in the blood of these cats after 10 days post inoculation and there were no obvious clinical signs throughout the sixty day observation period, and the

bacteria could not be isolated from the kidney post mortem (Ferris and Andrews 1965). Twenty years later, a similar study experimentally infecting cats via subcutaneous innoculation with *L.icterohaemorrhagiae* and *L.canicola,* extended the observation period to 77 and 84 days in two cats inoculated with the latter (Larsson et al. 1985). None of the cats elicited any clinical signs during the observation period, and bacterial cultures of the of the kidneys post mortem were negative for *Leptospira* in all cats. Others have also reported seropositivity amongst this species as having no clinical significance (Obrenovic et al. 2014, Mylonakis et al. 2005, Azocar-Aedo, Monti and Jara 2014).

Although the current study failed to demonstrate a significant relationship between CKD in cats and leptospiral positivity by PCR of the blood, it does not rule out leptospiral infection as a cause of CKD. The presence of *Leptospira* in the blood would imply active infection without an effective initiated immune response (Levett 2001). This study found a significant correlation between AKI and a positive blood sample, as well as younger cats being more likely to demonstrate a PCR positivity (Two-tailed P value=0.016) (Section 3.4.1, Table 3-6). It is possible that cats become infected with leptospiral bacteria at a younger age, and the initial insult to the kidneys further develops throughout the lifespan of the cat, despite the development of an effective immune response clearing the initial infection and possible future transient infections, progressing into CKD as a geriatric. It could also be that, once the bacteria has been cleared from the blood, it colonises in the kidney tissue provoking continuous injury. In future investigations, it would be useful to sample the kidney tissue for isolation of the bacteria in order to explore this further. This was not possible during this study. Collection of urine samples for PCR testing, would also have given an indication of presence of bacteria within the kidneys, but due to

storage conditions and timings, this also was not possible but to be considered in the future. Rodriguez et al. also encouraged use of multiple diagnostic tests to enable a clearer picture of leptospiral infection in cats to be formed (Rodriguez et al. 2014).

4.2 Investigating the association between demographic variables and leptospiral positivity

Leptospiral positivity by PCR of the blood was significantly higher amongst younger cats in this investigation with a two-tailed P value of 0.016 (Section 3.4.1, Table 3-6) which was not found in relation to PCR of urine or seropositivity in the cats in a similar study . It was also found that younger cats were significantly more susceptible to acute kidney injury in the current investigation with a P value 0.002 (Section 3.4.6, Table 3-21). It could be possible that younger cats, whose kidneys are less developed, are more susceptible to bacterial invasion and subsequent kidney damage, which then facilitates the further adhesion of bacteria until an efficient immune response clears the bacteria from the body. Once this immune response has been initiated, the adapted defence mechanisms may then be efficient enough to avoid future infection by the bacteria which may be supported by other studies that have found a higher amount of leptospiral antibodies in older cats (Mylonakis et al. 2005, Rodriguez et al. 2014). It is possible that initial kidney damage remains and progresses into CKD, most commonly found in geriatric cats. This would explain a negative PCR result for *Leptospira* in blood as there is no longer an active infection present in these older cats.

Rodriguez et al. found that cats living with another cat in the household were more likely to be seropositive and it was suggested that the sharing of a litter tray may have been the transmission source (Rodriguez et al. 2014). There was no significant connection between PCR positivity in the current study and the presence of another cat in the household (Section 3.4.1, Table 3-7). It is not known how many of these cats have outdoor access and it would be assumed that toileting outside would

reduce this suggested transmission risk. However, it would also be expected that those cats that have outdoor access may have the added risk of contracting the infection from external environmental sources and from hunting infected prey (Schuller et al. 2015, Rodriguez et al. 2014), but access to the outdoors was not found to be significant in predicting leptospiral positivity (Section 3.4.1, Table 3-7).

It is not surprising that this study did not find the presence of a dog in the household as significant (Section 3.4.1, Table 3-7), because although they would be living in close proximity of each other and leptospirosis is a common disease of the canine species, the majority of dogs tend to be vaccinated against it, with a recent report revealing 79% of pet dogs receive regular booster vaccinations in the UK (Anonymous 2017). There was also no significant correlation between the presence of a dog in the household with the seroprevalence of 124 domestic cats in Chile (Azocar-Aedo et al. 2014).

It must also be considered that due to inconsistencies in types of questions asked by differing clinicians to the owners, a lot of the information regarding demographic variables was missing (Section 3.4.1, Table 3-4) making these sample numbers smaller, reducing the reliability of results from statistical analysis.

4.3 Investigating the associate between laboratory values and leptospiral positivity

4.3.1 Haematological values

When reviewing various published literature, the most common haematological findings in canine leptospirosis were neutrophilia, lymphopenia of varying degree and non-regenerative anaemia (Sykes et al. 2011). A European consensus statement agreed with these findings and also found monocytosis to be a common abnormality in canine infection (Schuller et al. 2015). Both literature reviews agreed that thrombocytopenia was another reason for differential diagnosis of leptospiral infection, when accompanied by acute kidney injury. Kohn et al. found that 58% of dogs with leptospirosis has thrombocytopenia reported on admission. De Silva et al. found thrombocytopenia in 80.7% at some stage of illness, with a peak being reported on the 5th day of fever (De Silva et al. 2014) when investigating human leptospiral infection. Its common occurrence amongst this species was also highlighted by others (Jaureguiberry et al. 2005, van de Werve et al. 2013). Anaemia and leucocytosis was reported in 50% or more of dogs at admission in another study (Kohn et al. 2010). The main findings of the current study associated a higher lymphocyte and basophil count, and eosinophilia, with leptospiral positivity amongst the cats investigated (Section 3.4.2).

There is very limited available information surrounding the haematological effect of feline leptospirosis and therefore it is difficult to draw an effective comparison to the current study. In one feline case report, cats with clinical leptospirosis demonstrated neutrophilia, with a left shift and an elevated haematocrit value, or a milder neutrophilia alongside lymphopenia and moderate thrombocytopenia (Arbour et al.

2012) with the serovar Pomona. Lymphocytosis has been found in one case study in feline clinical infection with serovars belonging to the sejroë serogroup (Beaudu-Lange and Lange 2014)

In the current study, cats with deemed leptospiral positivity by PCR had a significantly higher number of lymphocytes (average = 3.08 x 10^9/l) in the blood than leptospiral negative cats (average = 2.28 x 10^9/l) (Section 3.4.2, Table 3-9) with a two-tailed P value of 0.029 (Section 3.4.2, Table 3-10). Lymphocytosis (lymphocyte count above in the reference interval) was present in 2/24 (8.33%) and 0/134 (0%) of leptospiral positive and negative cats, respectively, and this was found to be significantly associated with leptospiral positivity with a two-tailed P value of 0.0223 (Section 3.4.2, Table 3-11). Lymphopenia was reported in 9/24 (37.5%) of positive cats and 70/134 (52.24%) of negative cats and was not found to be significant (Section 3.4.2, Table 3-11).

A consensus statement reported lymphopenia to be commonly associated with canine infection, and another study agreed with this and reported lymphocytosis in as little as 2% of canine patients (Kohn et al. 2010). Craig et. al measured lymphocytes in human patients at different stages of the disease and also found lymphopenia to be a common finding in patients with leptospirosis (Craig et al. 2009). All patients in the acute phase (before an effective immune response was initiated) demonstrated lymphopenia and this level of incidence was less in those that were at the start of the immune phase and decreased even further in those classed in the immune phase of the disease. This was thought to be due to lymphotoxic exoproducts produced by the circulating *Leptospira* until an effective immune response clears them from the blood. There is not enough evidence to

suggest how long each of the different stages of leptospiral infection last in a cat and whether this would explain the pattern of lymphopenia amongst the cats in the current study.

It could be that cats are repeatedly exposed to the bacterium, as demonstrated by varying seroprevalence worldwide (Section 1.2.1), allowing them to build up an effective immune response. An immune response that quickly recognises and acts against the *Leptospira* during infection would limit the period of opportunity for the bacterium to release the lymphotoxic substances, before being destroyed themselves by the leukocytes.

The current study also showed that leptospiral positive cats had a significantly higher basophil count compared to the leptospiral negative cats with a P value of 0.032 (Section 3.4.2, Table 3-10). The mean basophil count was 0.09 x 10^9/l and 0.02 x 10^9/l in the positive and negative cats, respectively (Section 3.4.2, Table 3-9). 3/24 (12.5%) of the positive cats and 5/134 (3.73%) of the negative cats had a basophil count value above that of the normal range (0-0.1 x 10^9/l), and this was not found to be significant (Section 3.4.2, Table 3-11). Basophils have mainly been linked to allergic, autoimmune and cancerous processes as well as organ rejection (Siracusa et al. 2013) so its association with leptospirosis is unclear and has not been reported in any other literature, with the exception of one equine study that found the level of basophils in horses with leptospirosis remained higher than that of healthy horse, within the first 5 weeks of the study, however the relevance of this was not reported (Sohail et al. 2017). The lack of association with an *abnormally* high basophil count and leptospiral positivity also puts the relevance of this general association into question.

There was also a significant association (two-tailed P value = 0.0203) found between eosinophilia and leptospiral positivity with 5/24 (20.83%) and 7/134 (5.22%) of leptospiral positive and leptospiral negative cats, respectively, demonstrating a value above the normal reference interval (Section 3.4.2, Table 3-11). One canine study found eosinophilia in only 2% of dogs with leptospirosis (Kohn et al. 2010). One clinical review compared eosinophils to neutrophils in that the respond to infection and initiate phagocytosis of bacterium (Ravin and Loy 2016) and this could explain the higher numbers seen in leptospiral infection.

When analysing the platelet counts of the cats in the current study, there was no significant association found between this and leptospiral positivity when using the binary logistic regression analysis (Section 3.4.2, Table 3-10), nor when analysing platelet counts outside the RI using the two-tailed Fisher's exact test (Section 3.4.2, Table 3-11). This contrasts to human and canine studies which have reported thrombocytopenia to be a common finding in leptospirosis (De Silva et al. 2014, Kohn et al. 2010, van de Werve et al. 2013, Jaureguiberry et al. 2005). Kohn et al. found this more commonly in dogs with pulmonary abnormalities secondary to leptospirosis (Kohn et al. 2010) whilst another study associated it with severe disease (Hochedez et al. 2015). Neutrophilia has also been associated as a clinical manifestation of severe disease in another study (De Silva et al. 2014), as well has having been reported as a common finding in canine infection (Kohn et al. 2010, Prescott et al. 2002). There was no significant relationship between cats deemed leptospiral positive and neutrophilia in the current study (Section 3.4.2, Table 3-11). Additionally, when performing the binary logistics regression analysis on the haematological values of cats in the current study, there was also no significant association found between neutrophil count and leptospiral positivity with a two-tailed

P value of 0.970 (Section 3.4.2, Table 3-10). Only two of the cats from the sample number of the current study were suspected as having a possible leptospiral infection by the leading clinician during their stay in the hospital and sent for diagnostic evaluation of this, independent of this study. This would imply their symptoms were severe enough to raise suspicion and require testing for the disease, and although other cats were found to be leptospiral positive using the qPCR in this investigation, the disease progression may not have been as serious. however, when analysing the individual haematology results of these cats (results not shown), neither had neutrophilia or thrombocytopenia. It could therefore be that, unlike in canine infection, thrombocytopenia and neutrophilia are not a common consequence of feline leptospirosis, although there is not enough literature to compare this to.

One study hypothesised that the effect on the vascular system may depend on the infecting serovar (Craig et al., 2013) and stage of disease (Craig et al. 2009). With limited information available of disease stage and progression in cats with leptospirosis, as well as the lack infecting serovar identification in the current study, it is difficult to explain the haematological changes seen in the cats included in this current investigation.

4.3.2 Biochemical values

Common biochemical findings in canine leptospirosis tend to be those indicating kidney and liver dysfunction, with the larger majority of cases showing elevated serum and creatinine levels (Sykes et al. 2011). The current study found a significant correlation between alanine aminotransferase (ALT) levels, total protein levels below the RI and thyroxine levels above the RI, with leptospiral positivity, as well as thyroxine levels below the RI have a significant correlation to leptospiral negativity (Section 3.4.3).

Although the leptospiral positive cats in the current study showed a higher mean value of serum urea and creatinine values than that of the PCR negative cats (Section 3.4.3, Table 3-12), despite a reasonably strong positive correlation found between serum creatine levels and leptospiral positivity with a P value of 0.073 (Section 3.4.3, Table 3-13), this was not found to be significant. Serum creatinine and urea values outside of the reference intervals for these biochemical measurements also showed no significant correlation to leptospiral positivity (Section 3.4.3, Table 3-14). This is interesting as AKI was found to be significantly associated with leptospiral positivity with a P value of 0.0192 (Section 3.4.6, Table 3-20) and increased serum creatinine levels are usually the main indicator of this state (Ronco, Kellum and Haase 2012). Arbour et al. reported one cat with clinical leptospirosis as having normal serum creatinine levels with a lightly elevated urea (Arbour et al. 2012), despite demonstrating a very high titre of 1/12,800 for *L.* Pomona. This cat responded well to treatment and survived. The second cat described in the same paper presented with creatinine values within the reference range and a high MAT titre for *L.* Pomona and also survived after treatment. The third cat, however,

presented with severely elevated urea and creatinine levels, and despite treatment, deteriorated and was euthanased, *Leptospira* was confirmed by MAT and PCR on urine and renal tissue. The first two cats that responded to treatment had been showing clinical signs for no longer than two weeks, the third had been showing them for a few months. Hugely elevated creatinine levels may therefore be associated with the final stages of the disease, and it may take a few months to progress to this stage.

A significant positive correlation of a P value of 0.022 (Section 3.4.3, Table 3-13) was found in the current study between Alanine aminotransferase (ALT) levels and leptospiral positivity on PCR when performing a binary logistic regression analysis. 17/24 (70.83%) of the leptospiral positive cats and 98/134 (73.13%) of the negative cats had an ALT value above that of the laboratory reference range (14-45 U/l), although this was not found to be significant (Section 3.4.3, Table 3-14). In human medicine, abnormal serum ALT levels are considered a good indicator of liver disease (Kim et al. 2008). Mild increases, however tend to be ignored as are not considered clinically significant. Increases in ALT are less frequently seen than that of ALP and total bilirubin when analysing the serum biochemistry of dogs with leptospirosis (Schuller et al. 2015) and therefore its relevance in this study, when unaccompanied by a significant increase ALP or bilirubin, is unknown. ALT is released from the liver as a consequence of hepatocyte injury or expiration (Kim et al. 2008).

Liver dysfunction has been recognised in reported cases of feline leptospirosis, including indications such as the development of icterus (Mason et al. 1972, Rees 1964) and the icteric form of leptospirosis has been acknowledged as a very severe

and progressive from of the disease (Levett 2001). Post mortem examination of liver demonstrated abnormalities such as gross enlargement, centrinobular necrosis, hepatic amyloidosis, parenchymal fatty changes or noted friability of the parenchyma (Bryson and Ellis 1976, Mason et al. 1972, Harkness et al. 1970). One study noted elevated liver enzymes in the majority of seropositive cats (Agunloye and Nash 1996) with no obvious clinical signs related to this. It was hypothesised that elevated liver enzymes may be an early indicator of the disease and development of jaundice may occur in the final stages. In agreement with this idea, others have reported cause for euthanasia at this stage, as well as one cat dying due to unsuccessful attempts at treatment (Mason et al. 1972, Rees 1964). Icterus may also be related to *L.* Pomona infection (Bryson and Ellis 1976). However, some cats that have been highly seropositive to *L.* Pomona have shown no obvious liver implications (Arbour et al. 2012), as well as some experimentally infected with this serovar (Fessler and Morter 1964), but this could also be due to infection stages. Unfortunately, the *Leptospira* in the current study was not speciated to enable comparison.

Another finding in the current study was a significant association between total protein value below the RI (77-91 g/l) and leptospiral negativity with a two-tailed P value of 0.0290 (Section 3.4.3, Table 3-14). This consisted of 19/23 (82.6%) of the positive cats and 126/131 (96.18%), with a total of 145/154 (94.16%) of all cats. It was difficult to find reason for this association but due to the large proportion of total cats with an abnormally low total protein value, this may indicate incorrect reference range of this test.

9/13 (69.23%) leptospiral positive cats and 28/85 (32.94%) of leptospiral negative cats were reported to have thyroxine levels above the RI (15-60mmol/l) at the time of

blood sampling, confirming a significant positive correlation between biochemical hyperthyroidism and leptospiral positivity with a two-tailed P value of 0.0279 (Section 3.4.3, Table 3-14). Biochemical hypothyroidism was reported in 0/13 (0%) and 25/85 (29.4%) of leptospiral positive and leptospiral negative cats, respectively, resulting in a significant positive correlation between hypothyroidism and leptospiral negativity (Section 3.4.3, Table 3-14). These are novel and unexpected findings and there is no available literature to support them or help explain the mechanisms behind them.

A review identified other common clinical biochemical findings, such as potassium and phosphate/phosphorus levels below and above normal, and abnormally low sodium and chloride levels (Schuller et al. 2015). None of these values were found to have a positive correlation to the leptospiral positive cats in the current study (Section 3.4.3, Table 3-13 and Table 3-14). This could be an indication of disease progression, or a difference in clinical manifestation between the infected species.

4.3.3 Urinalysis values

Reviews of literature concerning canine leptospirosis confirm isothenuria and proteinuria as a common finding on urinalysis, as well as the presence of glucose and blood in the urine (Sykes et al. 2011, Schuller et al. 2015) but there is little evidence of urinary changes in cats infected with the bacteria. One study experimentally infected cats with *L.interrogans* and *L.canicola* and, in the 56 days post inoculation, no changes in urinary values were noted (Larsson et al. 1985) , although this could be due to incubation periods being longer to that of the observation period used in the experiment or serovar-specific effects on urine.

There were no significant associations found between any of the urine values and leptospiral positivity by PCR in this study (Section 3.4.4, Table 3-17).

4.3.4 Virology and serology variables

There were no significant associations reported between leptospirosis and FeLV or FIV in this investigation (Section 3.4.5, Table 3-19). However, there was also a very low positivity reported of cats with these diseases and therefore not a large enough number to draw a reliable conclusion from.

There was also not a significant association reported between a positive MAT result and for *Leptospira* and a positive PCR result (Section 3.4.5, Table 3-19). This is due to the fact that only 2/158 cats were tested for the presence of *Leptospira* by MAT and neither were deemed positive by this diagnostic method. This also highlights the lack of investigation into acute leptospiral infection as a cause of kidney dysfunction in cats as only 1/8 cats included in this study with AKI noted in the case report were tested for the bacteria. However, 3/8 of these cats were reported as either having ingested a nephrotoxic substance (n=1) or current urolithiasis or nephrolithiasis (n=2) which may have been causing the kidney damage.

4.3.5 Other significant findings

This study showed a significant positive correlation between cats with current urolithiasis or nephrolithiasis and leptospiral positivity demonstrated by a two-tailed P value of 0.0344 (Section 3.4.6, Table 3-20). It was hypothesised that the pH changes involved that may encourage stone formation in the urinary system may also favour the conditions for leptospiral survival and colonisation. A lower urinary pH optimises uric acid stone formation (Maalouf et al. 2004) and calcium oxalate crystal formation (Cottam et al. 2002), whereas a urinary pH above 6.6 increases likelihood of struvite crystal form.

In a study that analyses the movement of a saprophytic strain of *Leptospira*, motility of the bacteria increased in more alkaline environments (Islam et al. 2015). Lin et al investigated the effect pH has on the binding of the leptospiral immunoglobin-like proteins to the extracellular matrix components of host tissue cells and optimal pH for certain protein binding was between 4.5-8.5 (Lin et al. 2009). The acidic conditions within the urinary system that initiate some stone formation also reduce the motility of the *Leptospira* which could create more of an opportunity and time to bind to adjacent tissues within the kidneys. The range in pH optimal for binding of certain proteins means this environment would be favourable to tissue invasion.

However, when analysing the urinary pH of the cats included in this study (Section 3.4.4, Table 3-16), it was found that those with urolithiasis or nephrolithiasis reported at the time of blood sampling had an average urinary pH of 6.77 (range: 6.1-6.7) and those without had an average urinary pH of 6.68 (range: 4.5-8.7). With very little difference between the urinary pH between these two groups, it does not explain the reported correlation.

It is also a possibility that stone formation within the urinary tract is secondary to leptospiral infection. The urinary pH of those with mild leptospirosis (5.34 ± 0.22) and severe leptospirosis (5.55 ± 051) were both lower than those suffering from acute tubular necrosis in a study analysing the haematological and urinary findings in humans patients (Abdulkader et al. 1996). Although the mechanism for this effect on urinary pH is unclear, these acidic conditions could predispose these patients to urolithiasis/nephrolithiasis and the same could be assumed for the feline population in the current study. However, in the current study, the average urinary pH of cats that test leptospiral positive by PCR was 6.74 which was very similar to those which tested negative for leptospiral DNA in the blood which was 6.68 (Section 3.4.4, Table 3-15). It is therefore difficult to understand the mechanisms behind this relationship. There was also no correlation found between cats with a previous history of urolithiasis and nephrolithiasis (excluding those with current stones reported) and leptospiral positivity (Section 3.4.6, Table 3-20).

Unfortunately, due to storage conditions and timing restrictions, PCR analysis could not be performed on the urine of the cats and therefore evidence of the presence and colonisation of the bacteria within the kidneys could not be investigated. Nor was kidney sampling feasible or ethical in this retrospective study.

5 Conclusion

When analysing 158 cats, the current investigation found no significant association between chronic kidney disease in cats and leptospiral infection when using qPCR on whole blood samples (Section 3.3). However, by analysing a whole blood sample, a positive result would indicate the presence of leptospiraemia, an active infection in the blood before an effective immune response has been initiated. This study found a positive correlation between leptospiral positivity and AKI (Section 3.4.6) and therefore, it is still possible that this original insult could still cause enough kidney damage to go on to progress into chronic kidney disease, despite there not being evidence of active infection amongst these cats with the chronic condition. A leptospiral positive PCR result was also higher amongst the younger cats, which would support this idea, as at a younger age cats are more susceptible to novel infection by the bacteria (Section 3.4.1) before building up an effective immune response to target infection in the future. Therefore, *Leptospira* could still be one of the unknown causes of the common geriatric disease in cats. It is important to note that the IRIS staging of CKD usually requires the measurement of serum creatinine on at least two separate occasions (IRIS 2015). It was only possible to acquire one measurement of the cats included in this study, which could have led to the misdiagnosis of CKD.

Overall, 24/158 (15.19%) cats were found to have a positive PCR result for *Leptospira* and there were patterns reported amongst haematology and serum biochemistry test results in cats with leptospiral positivity (Sections 3.4.2 and 3.4.3). For instance, cats with leptospiral positivity had significantly higher basophil and lymphocyte counts, as well as a higher alanine aminotransferase value in the serum.

When looking at patterns in values outside of the normal reference interval, leptospiral positive cats were significantly more likely to have eosinophilia, lymphocytosis, biochemical hyperthyroidism and a low total protein count. There were no significant findings amongst the urinalysis results (Section 3.4.4).

There were also no significant findings amongst any of the demographic details recorded, including outdoor access, neuter or vaccination status, or presence of another dog or cat in the household (Section 3.4.1). Cats with current evidence of nephrolithiasis or urolithiasis were found to have a significant correlation to leptospiral positivity (Section 3.4.6), although the mechanism for this could not be explained.

Although there were significant findings amongst the blood and serum test results of the cats in this study, the relevance of these could be disputed. A large limitation of this investigation was that the cats included were patients at the veterinary hospital and were suffering from a wide range of different medical or surgical conditions, which would also influence these test results. In order to fully evaluate the clinical relevance of feline leptospiral infection, healthy cats would need to be infected and the haematological, biochemical and urinary values, as well as clinical signs, analysed and monitored over a period of time and compared to a control group. The longest observation period recorded amongst experimentally infected cats is currently 84 days (Larsson et al. 1985). Since so little is known about leptospiral infection of cats, it would be ideal to extend this observation time and also to infect cats with different serovars and study the effects this may have. Post mortem analysis would also prove useful to evaluate direct effects the *Leptospira* may have on the organs.

It is apparent from case studies that the serovar Pomona may cause clinical disease in cats (Mason et al. 1972, Arbour et al. 2012, Rees 1964, Harkness et al. 1970), and has also been recently isolated from, and highlighted as a potential health threat to, livestock in the UK (Arent et al. 2017), and therefore may be of particular interest for future study of feline infection, as well as the potential for transmission risk.

Another limitation of the current study was the ability to perform qPCR of whole blood samples only. Unfortunately, due to storage timings, and the ability for DNA degradation in urine at various temperatures (Morre et al. 1999, Cannas et al. 2009, Palmirotta et al. 2011), it was decided that this specimen type would not be used for PCR analysis in the current study. This analysis would provide more information on transmission risk from this domesticated species. PCR analysis of kidney tissue would also help evaluate the presence of *Leptospira* in renal tissue, especially when investigating the role of the bacterium in chronic kidney disease, however the sampling of this was not ethically feasible in the current study. All of these tests run together would help to paint a bigger picture of feline infection with *Leptospira* and should be considered to be performed in conjunction with each other in future experiments.

Although, this study failed to demonstrate an association with leptospiral positivity and CKD in cats, its association with AKI could imply this acute condition is a clinical manifestation of the infection in cats. Younger cats were also found more likely to be leptospiral positive. It is possible that younger cats are more susceptible to infection by the bacterium and this can cause acute kidney damage before an effective immune response is elicited and prevents future infection. This initial insult to the kidneys may well progress into CKD as a cat gets older, despite there not being

evidence of active letpospiraemia at the time for diagnosis of the chronic condition, and therefore leptospiral infection may still be of aetiological relevance to CKD in cats.

6 Appendix 1

1. NAME OF THE VETERINARY MEDICINAL PRODUCT

Nobivac L4, suspension for injection for dogs

2. QUALITATIVE AND QUANTITATIVE COMPOSITION

Each dose of 1 ml contains:

Active substances:
Inactivated *Leptospira* strains:
- *L. interrogans* serogroup Canicola serovar Portland-vere (strain Ca-12-000) 3550-7100 U[1]
- *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni (strain Ic-02-001) 290-1000 U[1]
- *L. interrogans* serogroup Australis serovar Bratislava (strain As-05-073) 500-1700 U[1]
- *L. kirschneri* serogroup Grippotyphosa serovar Dadas (strain Gr-01-005) 650-1300 U[1]

[1] Antigenic mass ELISA units.

Excipient:
Thiomersal 0.1 mg

For a full list of excipients, see section 6.1.

3. PHARMACEUTICAL FORM

Suspension for injection
Colourless suspension

4. CLINICAL PARTICULARS

4.1 Target species

Dogs

4.2 Indications for use, specifying the target species

For active immunisation of dogs against:
- *L. interrogans* serogroup Canicola serovar Canicola to reduce infection and urinary excretion
- *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni to reduce infection and urinary excretion
- *L. interrogans* serogroup Australis serovar Bratislava to reduce infection
- *L. kirschneri* serogroup Grippotyphosa serovar Bananal/Lianguang to reduce infection and urinary excretion

Onset of immunity: 3 weeks
Duration of immunity: 1 year

7 References

Abdulkader, R., A. C. Seguro, P. S. Malheiro, E. A. Burdmann & M. Marcondes (1996) Peculiar electrolytic and hormonal abnormalities in acute renal failure due to leptospirosis. *American Journal of Tropical Medicine and Hygiene,* 54**,** 1-6.

Acierno, M. J. & D. Senior. 2010. Urinary disorders. In *Clinical Medicine of the Dog and Cat,* ed. M. Schaer, 514. London: Manson Publishing.

Adams, W. H., G. B. Daniel & A. M. Legendre (1997a) Investigation of the effects of hyperthyroidism on renal function in the cat. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire,* 61**,** 53-56.

Adams, W. H., G. B. Daniel, A. M. Legendre, R. E. Gompf & C. A. Grove (1997b) Changes in renal function in cats following treatment of hyperthyroidism using I-131. *Veterinary Radiology & Ultrasound,* 38**,** 231-238.

Agampodi, S. B., M. A. Matthias, A. C. Moreno & J. M. Vinetz (2012) Utility of Quantitative Polymerase Chain Reaction in Leptospirosis Diagnosis: Association of Level of Leptospiremia and Clinical Manifestations in Sri Lanka. *Clinical Infectious Diseases,* 54**,** 1249-1255.

Agunloye, C. A. & A. S. Nash (1996) Investigation of possible leptospiral infection in cats in Scotland. *Journal of Small Animal Practice,* 37**,** 126-129.

Ahmad, S. N., S. Shah & F. M. Ahmad (2005) Laboratory diagnosis of leptospirosis. *J Postgrad Med,* 51**,** 195-200.

Ahmed, A., M. F. M. Engelberts, K. R. Boer, N. Ahmed & R. A. Hartskeerl (2009) Development and Validation of a Real-Time PCR for Detection of Pathogenic Leptospira Species in Clinical Materials. *Plos One,* 4.

Anitha, S., G. D. Raj, A. Raja & A. Thangavelu (2012) Comparison of DNA extraction methods and gene targets from Leptospira interrogans serovar icterohaemorrhagiae for application in polymerase chain reaction. *Indian Journal of Animal Sciences,* 82**,** 1133-1136.

Anonymous. 2017. PAW PDSA Animal Welbeing Report 2017. 11-35. People's Dispensary for Sick Animals.

Arbour, J., M. C. Blais, L. Carioto & D. Sylvestre (2012) Clinical Leptospirosis in Three Cats (2001-2009). *Journal of the American Animal Hospital Association,* 48**,** 256-260.

Arent, Z., C. Gilmore, A. M. Barlow, L. Smith & W. A. Ellis (2017) Leptospira interrogans serogroup Pomona infections in the UK: is there a real threat for farm animals? *Veterinary Record,* 180**,** 424.

Azocar-Aedo, L., G. Monti & R. Jara (2014) Leptospira spp. in Domestic Cats from Different Environments: Prevalence of Antibodies and Risk Factors Associated with the Seropositivity. *Animals (Basel).* 4**,** 612-26. doi: 10.3390/ani4040612.

Bajani, M. D., D. A. Ashford, S. L. Bragg, C. W. Woods, T. Aye, R. A. Spiegel, B. D. Plikaytis, B. A. Perkins, M. Phelan, P. N. Levett & R. S. Weyant (2003) Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. *Journal of Clinical Microbiology,* 41**,** 803-809.

Bal, A. E., C. Gravekamp, R. A. Hartskeel, J. de Mezza-Brewster, H. Korver & W. J. Terpstra (1994) Detection of Leptospires in Urine by PCR for Early Diagnosis of Leptospirosis. *Journal of Clinical Microbiology,* 32**,** 1894-1989.

Balakrishnan, A. & K. J. Drobatz (2013) Management of Urinary Tract Emergencies in Small Animals. *Veterinary Clinics of North America-Small Animal Practice,* 43**,** 843-867.

Barber, P. (2003) Diagnosis and management of chronic renal failure in the cat. *In Practice,* 25**,** 306.

Barnett, J. K., D. Barnett, C. A. Bolin, T. A. Summers, E. A. Wagar, N. F. Cheville, R. A. Hartskeerl & D. A. Haake (1999) Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. *Infection and Immunity,* 67**,** 853-861.

Bartges, J. W. (2012) Chronic Kidney Disease in Dogs and Cats. *Veterinary Clinics of North America-Small Animal Practice,* 42**,** 669-692.

Bartlett, P. C., J. W. Van Buren, A. D. Barlett & C. Zhou (2010) Case-Control Study of Risk Factors Associated with Feline and Canine Chronic Kidney Disease. *Veterinary Medicine International,* 2010**,** 1-9.

Baxter, K. J., J. K. Levy, C. H. Edinboro, S. L. Vaden & M. B. Tompkins (2012) Renal Disease in Cats Infected with Feline Immunodeficiency Virus. *Journal of Veterinary Internal Medicine,* 26**,** 238-243.

Beaudu-Lange, C. & E. Lange (2014) Unusual clinical presentation of leptospirosis in a cat. *Revue Vétérinaire Clinique,* 49**,** 115-122.

Beck, C. & R. B. Lavelle (2001) Feline polycystic kidney disease in Persian and other cats: a prospective study using ultrasonography. *Australian Veterinary Journal,* 79**,** 181-184.

Becker, T. J., T. K. Graves, J. M. Kruger, W. E. Braselton & R. F. Nachreiner (2000) Effects of methimazole on renal function in cats with hyperthyroidism. *Journal of the American Animal Hospital Association,* 36**,** 215-223.

Bedir, O., A. Kilic, E. Atabek, A. M. Kuskucu, V. Turhan & A. C. Basustaoglu (2010) Simultaneous Detection and Differentiation of Pathogenic and Nonpathogenic Leptospira spp. by Multiplex Real-Time PCR (TaqMan) assay. *Polish Journal of Microbiology,* 59**,** 167-173.

Berent, A. C. (2011) Ureteral obstructions in dogs and cats: a review of traditional and new interventional diagnostic and therapeutic options. *Journal of Veterinary Emergency and Critical Care,* 21**,** 86-103.

Bourhy, P., S. Bremont, F. Zinini, C. Giry & M. Picardeau (2011) Comparison of Real-Time PCR Assays for Detection of Pathogenic Leptospira spp. in Blood and Identification of Variations in Target Sequences. *Journal of Clinical Microbiology,* 49**,** 2154-2160.

Boyce, J. T., S. P. Dibartola, D. J. Chew & P. W. Gasper (1984) Familial renal amyloidosis in Abyssinian cats. *Veterinary Pathology,* 21**,** 33-38.

Boyd, L. M., C. Langston, K. Thompson, K. Zivin & M. Imanishi (2008) Survival in cats with naturally occurring chronic kidney disease (2000-2002). *Journal of Veterinary Internal Medicine,* 22**,** 1111-1117.

Branger, C., B. Blanchard, C. Fillonneau, I. Suard, F. Aviat, B. Chevallier & G. Andre-Fontaine (2005) Polymerase chain reaction assay specific for pathogenic Leptospira based on the gene hap1 encoding the hemolysis-associated protein-1. *Fems Microbiology Letters,* 243**,** 437-445.

Brown, P. D., C. Gravekamp, D. G. Carrington, H. van de Kemp, R. A. Hartskeerl, C. N. Edwards, C. O. Everard, W. J. Terpstra & P. N. Levett (1995) Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. *J Med Microbiol,* 43**,** 110-4.

Bryson, D. G. & W. A. Ellis (1976) Leptospirosis in the Bristish domestic cat. *Journal of Small Animal Practice,* 17**,** 459-465.

Cai, H. Y., G. Hornby, D. W. Key, M. R. Osuch & M. G. Maxie (2002) Preliminary study on differentiation of Leptospira grippotyphosa and Leptospira sejroe from other common pathogenic leptospiral serovars in canine urine by polymerase chain reaction assay. *Journal of Veterinary Diagnostic Investigation,* 14**,** 164-168.

Cannas, A., G. Kalunga, C. Green, L. Calvo, P. Katemangwe, K. Reither, M. D. Perkins, L. Maboko, M. Hoelscher, E. A. Talbot, P. Mwaba, A. I. Zumla, E. Girardi, J. F. Huggett & T. B. T. Consortium (2009) Implications of Storing Urinary DNA from Different Populations for Molecular Analyses. *Plos One,* 4.

Cermakova, Z., P. Kucerova, L. Pliskova & P. Kubickova (2013) Real-time PCR method for the detection of the gene encoding surface lipoprotein LipL32 of pathogenic Leptospira: use in the laboratory diagnosis of the acute form of leptospirosis. *Scandinavian Journal of Infectious Diseases,* 45**,** 593-599.

Chams, H., M. Rostami, S. F. Mohammadi & S. Ohno (2009) Epidemiology and Prevalence of Uveitis: Review of Literature. *Iranian Journal of Ophthalmology,* 21**,** 4-16.

Cheema, P. S., S. K. Srivastava, R. Amutha, S. Singh, H. Singh & M. Sandey (2007) Detection of pathogenic leptospires in animals by PCR based on lipL21 and lipL32 genes. *Indian Journal of Experimental Biology,* 45**,** 568-573.

Clark, C. H. (1977) Toxicitiy of aminoglycoside antibiotics. *Modern Veterinary Practice,* 58**,** 594-598.

Cottam, Y. H., P. Caley, S. Wamberg & W. H. Hendriks (2002) Feline reference values for urine composition. *Journal of Nutrition,* 132**,** 1754S-1756S.

Craig, S. B., G. C. Graham, M. A. Burns, M. F. Dohnt, L. D. Smythe & D. B. McKay (2009) Lymphopenia is observed regularly in the acute (leptospiraemic) phase but not the immune phase of leptospirosis. *Trans R Soc Trop Med Hyg.,* 103**,** 958-60. doi: 10.1016/j.trstmh.2009.03.019. Epub 2009 Apr 21.

De Silva, N. L., M. Niloofa, N. Fernando, L. Karunanayake, C. Rodrigo, H. J. De Silva, S. Premawansa, S. M. Handunnetti & S. Rajapakse. 2014. Changes in full blood count parameters in leptospirosis: a prospective study. In *Int Arch Med*, 31. doi: 10.1186/1755-7682-7-31

Dokuzeylul, B., A. Kayar & M. E. Or (2016) Prevalence of systemic disorders in cats with oral lesions. *Veterinarni Medicina,* 61**,** 219-223.

Dong, H. Y., Y. Hu, F. Xue, D. Sun, D. M. Ojcius, Y. F. Mao & J. Yan (2008) Characterization of the ompL1 gene of pathogenic Leptospira species in China and cross-immunogenicity of the OmpL1 protein. *Bmc Microbiology,* 8**,** 223.

Ehsanollah, S. & R. A. Gholam (2011) Detection of leptospiral antibodies by microscopic agglutination test in north-east of Iran. *Asian Pac J Trop Biomed,* 1**,** 227-9.

Elliott, J. & P. J. Barber (1998) Feline chronic renal failure: clinical findings in 80 cases diagnosed between 1992 and 1995. *Journal of Small Animal Practice,* 39**,** 78-85.

Ellis, W. A. (2010) Control of canine leptospirosis in Europe: time for a change? *Veterinary Record,* 167**,** 602-605.

---. 2014. Animal Leptospirosis. In *Leptospira and Leptospirosis,* ed. B. Adler, 99-138. Verlag Berlin Heidelberg: Springer.

Evangelista, K. V. & J. Coburn (2010) Leptospira as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. *Future Microbiol,* 5**,** 1413-25.

Faber, N. A., M. Crawford, R. B. LeFebvre, N. C. Buyukmihci, J. E. Madigan & N. H. Willits (2000) Detection of Leptospira spp. in the aqueous humor of horses with naturally acquired recurrent uveitis. *Journal of Clinical Microbiology,* 38**,** 2731-2733.

Ferris, D. H. & R. D. Andrews (1965) Leptospira Pomona in the feral cat. *American Journal of Veterinary Research,* 26**,** 373-376.

Fessler, J. F. & R. L. Morter (1964) Experimental feline leptospirosis. *Cornell Veterinarian,* 54**,** 176-190.

Finch, N. (2014) Measurement of glomerular filtration rate in cats: Methods and advantages over routine markers of renal function. *Journal of Feline Medicine and Surgery,* 16**,** 736-748.

Fink, J. M., G. E. Moore, R. Landau & R. Vemulapalli (2015) Evaluation of three 5 ' exonuclease-based real-time polymerase chain reaction assays for detection of pathogenic Leptospira species in canine urine. *Journal of Veterinary Diagnostic Investigation,* 27**,** 159-166.

Fitzgerald, K. T. (2010) Lily Toxicity in the Cat. *Topics in Companion Animal Medicine,* 25**,** 213-217.

Gabor, L. J., P. J. Canfield & R. Malik (2000) Haematological and biochemical findings in cats in Australia with lymphosarcoma. *Australian Veterinary Journal,* 78**,** 456-461.

Gabor, L. J., R. Malik & P. J. Canfield (1998) Clinical and anatomical features of lymphosarcoma in 118 cats. *Australian Veterinary Journal,* 76**,** 725-732.

Gomes, V. D., P. C. Ariza, N. C. Borges, F. J. Schulz & M. Fioravanti (2018) Risk factors associated with feline urolithiasis. *Veterinary Research Communications,* 42**,** 87-94.

Gouvela, E. L., J. Metcalfe, A. L. F. de Carvalho, T. S. F. Aires, J. C. Villasboas-Bisneto, A. Queirroz, A. G. Santos, K. Salgado, M. G. Reis & A. I. Ko (2008) Leptospirosis-associated severe pulmonary hemorrhagic syndrome, Salvador, Brazil. *Emerging Infectious Diseases,* 14**,** 505-508.

Gowan, R. A., A. E. Lingard, L. Johnston, W. Stansen, S. A. Brown & R. Malik (2011) Retrospective case-control study of the effects of long-term dosing with meloxicam on renal function in aged cats with degenerative joint disease. *Journal of Feline Medicine and Surgery,* 13**,** 752-761.

Grauer, G. F. (2005) Early detection of renal damage and disease in dogs and cats. *Veterinary Clinics of North America-Small Animal Practice,* 35**,** 581-596.

--- (2015) Laboratory Evaluation in Dogs & Cats with Chronic Kidney Disease. *Clinician's Brief,* 13**,** 65-69.

Gravekamp, C., H. Van de Kemp, M. Franzen, D. Carrington, G. J. Schoone, G. J. Van Eys, C. O. Everard, R. A. Hartskeerl & W. J. Terpstra (1993) Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *Journal of General Microbiology,* 139**,** 1691-700.

Greco, D. S. (2001) Congenital and inherited renal disease of small animals. *Veterinary Clinics of North America-Small Animal Practice,* 31**,** 393-399.

Greene, J. P., S. L. Lefebvre, M. S. Wang, M. Y. Yang, E. M. Lund & D. J. Polzin (2014) Risk factors associated with the development of chronic kidney disease in cats evaluated at primary care veterinary hospitals. *Javma-Journal of the American Veterinary Medical Association,* 244**,** 320-327.

Haake, D. A., G. Chao, R. L. Zuerner, J. K. Barnett, D. Barnett, M. Mazel, J. Matsunaga, P. N. Levett & C. A. Bolin (2000) The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infection and Immunity,* 68**,** 2276-2285.

Haake, D. A., M. A. Suchard, M. M. Kelley, M. Dundoo, D. P. Alt & R. L. Zuerner (2004) Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. *Journal of Bacteriology,* 186**,** 2818-2828.

Hardy, M. L., R. C. Hsu & C. R. Short (1985) The nephrotoxic potential of gentamicin in the cat - enzymuria and alterations in urine concentrating. *Journal of Veterinary Pharmacology and Therapeutics,* 8**,** 382-392.

Harkin, K. R., Y. M. Roshto & J. T. Sullivan (2003) Clinical application of a polymerase chain reaction assay for diagnosis of leptospirosis in dogs. *Journal of the American Veterinary Medical Association,* 222**,** 1224-1229.

Harkness, A. C., B. L. Smith & G. F. Fowler (1970) An isolation of Leptospira serotype pomona from domestic cat. *N Z Vet J.,* 18**,** 175-6.

Hartmann, K., H. Egberink, M. G. Pennisi, A. Lloret, D. Addie, S. Belak, C. Boucraut-Baralon, T. Frymus, T. Gruffydd-Jones, M. J. Hosie, H. Lutz, F. Marsilio, K. Mostl, A. D. Radford, E. Thiry, U. Truyen & M. C. Horzinek (2013) Leptospira Species Infection in Cats: ABCD guidelines on prevention and management. *Journal of Feline Medicine and Surgery,* 15**,** 576-581.

Hochedez, P., R. Theodose, C. Olive, P. Bourhy, G. Hurtrel, N. Vignier, H. Mehdaoui, R. Valentino, R. Martinez, J. M. Delord, C. Herrmann, I. Lamaury, R. Cesaire, M. Picardeau & A. Cabie (2015) Factors Associated with Severe Leptospirosis, Martinique, 2010-2013. *Emerging Infectious Diseases,* 21**,** 2221-2224.

IRIS (2015) IRIS Staging of CKD (modified 2015). 1-8. http://www.iris-kidney.com/guidelines/staging.html (last accessed 03/08/18).

Islam, M. S., Y. V. Morimoto, S. Kudo & S. Nakamura (2015) H+ and Na+ are involved in flagellar rotation of the spirochete Leptospira. *Biochemical and Biophysical Research Communications,* 466**,** 196-200.

Jamshidi, S., M. A. Akhavizadegan, S. Bokaie, N. Maazi & A. G. Ali (2009) Serologic study of feline leptospirosis in Tehran, Iran. *Iranian Journal of Microbiology,* 1**,** 32-36.

Jaureguiberry, S., M. Roussel, G. Brinchault-Rabin, A. Gacouin, A. Le Meur, C. Arvieux, C. Michelet & P. Tattevin (2005) Clinical presentation of leptospirosis: a retrospective study of 34 patients admitted to a single institution in metropolitan France. *Clinical Microbiology and Infection,* 11**,** 391-394.

Johnston, L. 2011. Use of meloxicom for the long-term treatment of kidney disorders in cats. In *Google Patent,* ed. U. P. Application, 1-6. United States: Boehringer Ingelheim Vetmedica Gmbh.

Joseph, S., M. Mini, V. K. Sriram, R. Ambily, T. Aravindakshan & S. A. Kumar (2018) Evaluation of real-time PCR, MAT, and recombinant LipL32-based ELISA for the diagnosis of canine leptospirosis in a disease-endemic South Indian state, Kerala. *Turkish Journal of Veterinary & Animal Sciences,* 42**,** 191-197.

Kim, W. R., S. L. Flamm, A. M. Di Bisceglie, H. C. Bodenheimer & A. Public Policy Committee American (2008) Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. *Hepatology,* 47**,** 1363-1370.

King, A. M., T. Bartpho, R. W. Sermswan, D. M. Bulach, A. Eshghi, M. Picardeau, B. Adler & G. L. Murray (2013) Leptospiral outer membrane protein LipL41 is not essential for acute leptospirosis but requires a small chaperone protein, lep, for stable expression. *Infect Immun,* 81**,** 2768-76.

King, J. N., S. Tasker, D. A. Gunn-Moore, G. Strehlau & B. S. Grp (2007) Prognostic factors in cats with chronic kidney disease. *Journal of Veterinary Internal Medicine,* 21**,** 906-916.

Knopfler, S., A. Mayer-Scholl, E. Luge, R. Klopfleisch, A. D. Gruber, K. Nockler & B. Kohn (2017) Evaluation of clinical, laboratory, imaging findings and outcome in 99 dogs with leptospirosis. *Journal of Small Animal Practice,* 58**,** 582-588.

Kohn, B., K. Steinicke, G. Arndt, A. D. Gruber, B. Guerra, A. Jansen, B. Kaser-Hotz, R. Klopfleisch, F. Lotz, E. Luge & K. Nockler (2010) Pulmonary Abnormalities in Dogs with Leptospirosis. *Journal of Veterinary Internal Medicine,* 24**,** 1277-1282.

Korman, R. M. & J. D. White (2013) Feline CKD Current therapies - what is achievable? *Journal of Feline Medicine and Surgery,* 15**,** 29-44.

Kyles, A. E., E. M. Hardie, B. G. Wooden, C. A. Adin, E. A. Stone, C. R. Gregory, K. G. Mathews, L. D. Cowgill, S. Vaden, T. G. Nyland & G. V. Ling (2005) Clinical, clinicopathologic, radiographic, and ultrasonographic abnormalities in cats with ureteral calculi: 163 cases (1984-2002). *Javma-Journal of the American Veterinary Medical Association,* 226**,** 932-936.

Lapointe, C., I. Plamondon & M. Dunn (2013) Feline leptospirosis serosurvey from a Quebec referral hospital. *Canadian Veterinary Journal-Revue Veterinaire Canadienne,* 54**,** 497-499.

Larsson, C. E., C. A. S. Rosa, M. Larsson, E. H. Birgel, W. R. Fernandes & G. V. Paim (1985) Laboratory and clinical-features of experimental feline leptospirosis. *International Journal of Zoonoses,* 12**,** 111-119.

Lees, G. E. (1996) Congenital renal diseases. *Veterinary Clinics of North America-Small Animal Practice,* 26**,** 1379-1399.

Lees, G. E., S. A. Brown, J. Elliott, G. E. Grauer & S. L. Vaden (2005) Assessment and management of proteinuria in dogs and cats: 2004 ACVIM forum consensus statement (small animal). *Journal of Veterinary Internal Medicine,* 19**,** 377-385.

Levett, P. N. (2001) Leptospirosis. *Clinical Microbiology Reviews,* 14**,** 296-326.

Levett, P. N., R. E. Morey, R. L. Galloway, D. E. Turner, A. G. Steigerwalt & L. W. Mayer (2005) Detection of pathogenic leptospires by real-time quantitative PCR. *Journal of Medical Microbiology,* 54**,** 45-49.

Limmathurotsakul, D., E. L. Turner, V. Wuthiekanun, J. Thaipadungpanit, Y. Suputtamongkol, W. Chierakul, L. D. Smythe, N. P. J. Day, B. Cooper & S. J. Peacock (2012) Fool's Gold: Why Imperfect Reference Tests Are Undermining the Evaluation of Novel Diagnostics: A Reevaluation of 5 Diagnostic Tests for Leptospirosis. *Clinical Infectious Diseases,* 55**,** 322-331.

Lin, Y. P., D. W. Lee, S. P. McDonough, L. K. Nicholson, Y. Sharma & Y. F. Chang (2009) Repeated Domains of Leptospira Immunoglobulin-like Proteins Interact with Elastin and Tropoelastin. *Journal of Biological Chemistry,* 284**,** 19380-19391.

Lindo, J., P. D. Brown, I. Vickers, M. Brown, S. T. Jackson & E. Lewis-Fuller (2013) Leptospirosis and malaria as causes of febrile illness during a dengue epidemic in Jamaica. *Pathogens and Global Health,* 107**,** 329-334.

Lizer, J., M. Grahlmann, H. Hapke, S. Velineni, D. Lin & B. Kohn (2017) Evaluation of a rapid IgM detection test for diagnosis of acute leptospirosis in dogs. *Veterinary Record,* 180**,** 517.

Lizer, J., S. Velineni, A. Weber, M. Krecic & P. Meeus (2018) Evaluation of 3 Serological Tests for Early Detection Of Leptospira-specific Antibodies in Experimentally Infected Dogs. *Journal of Veterinary Internal Medicine,* 32**,** 201-207.

Maalouf, N. M., M. A. Cameron, O. W. Moe & K. Sakhaee (2004) Novel insights into the pathogenesis of uric acid nephrolithiasis. *Current Opinion in Nephrology and Hypertension,* 13**,** 181-189.

Markovich, J. E., L. Ross & E. McCobb (2012) The Prevalence of Leptospiral Antibodies in Free Roaming Cats in Worcester County, Massachusetts. *Journal of Veterinary Internal Medicine,* 26**,** 688-689.

Martin, L. E. R., K. T. Wiggans, S. A. Wennogle, K. Curtis, R. Chandrashekar & M. R. Lappin (2014) Vaccine-Associated Leptospira Antibodies in Client-Owned Dogs Leptospira Vaccine Responses in Dogs Martin et al. *Journal of Veterinary Internal Medicine,* 28**,** 789-792.

Mason, R. W., N. M. McLachlan & S. J. King (1972) Suspected leptospirosis in two cats. *Australian Veterinary Journal,* 48**,** 622-623.

Matsunaga, J., M. A. Barocchi, J. Croda, T. A. Young, Y. Sanchez, I. Siqueira, C. A. Bolin, M. G. Reis, L. W. Riley, D. A. Haake & K. I. Albert (2003) Pathogenic Leptospira species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. *Molecular Microbiology,* 49**,** 929-945.

McLoughlin, M. A., S. P. Dibartola, S. J. Birchard & D. G. Day (1993) Influence of systemic nonthyroidal illness on serum concentration of thyroxine in hyperthyroid. *Journal of the American Animal Hospital Association,* 29**,** 227-234.

Meenambigai, T. V., G. Ravikumar, A. Srithar, G. Balkrishnan, C. Saranya & B. Muralimanohar (2011) Simultaneous detection of LipL32 and LipL21 genes of pathogenic leptospira from serum samples of bovines by multiplex PCR. *Veterinary Science Development,* 1**,** 60-62.

Merien, F., P. Amouriaux, P. Perolat, G. Baranton & I. Saintgirons (1992) Polymerase chain-reaction for detection of leptospira spp in clinical samples. *Journal of Clinical Microbiology,* 30**,** 2219-2224.

Minkus, G., C. Reusch, A. Horauf, W. Breuer, J. Darbes, W. Kraft & W. Hermanns (1994) Evaluation of renal biopsies in cats and dogs histopathology in comparison with clinical data. *Journal of Small Animal Practice,* 35**,** 465-472.

Morre, S. A., I. G. van Valkengoed, A. de Jong, A. J. Boeke, J. T. van Eijk, C. J. Meijer & A. J. van den Brule (1999) Mailed, home-obtained urine specimens: a reliable screening approach for detecting asymptomatic Chlamydia trachomatis infections. *J Clin Microbiol.,* 37**,** 976-80.

Mugford, A., R. Li & K. Humm (2013) Acute kidney injury in dogs and cats 1. Pathogenesis and diagnosis. *In Practice,* 35**,** 253-264.

Munday, J. S., D. J. Bergen & W. D. Roe (2005) Generalized calcinosis cutis associated with probable leptospirosis in a dog. *Veterinary Dermatology,* 16**,** 401-406.

Musso, D. & B. La Scola (2013) Laboratory diagnosis of leptospirosis: A challenge. *Journal of Microbiology Immunology and Infection,* 46**,** 245-252.

Mylonakis, M. E., E. Bourtzi-Hatzopoulou, A. F. Koutinas, E. Petridou, M. N. Saridomichelakis, L. Leontides & A. Siochu (2005) Leptospiral seroepidemiology in a feline hospital population in Greece. *Veterinary Record,* 156**,** 615-616.

Nash, K., A. Hafeez & S. Hou (2002) Hospital-acquired renal insufficiency. *American Journal of Kidney Diseases,* 39**,** 930-936.

Natarajaseenivasan, K., P. Vijayachari, S. Sharma, A. P. Sugunan & S. C. Sehgal (2005) Phenotypic & genotypic conservation of ompL1 & lipL41 among leptospiral isolates of Andaman Islands. *Indian Journal of Medical Research,* 122**,** 343-347.

Niloofa, R., N. Fernando, N. L. de Silva, L. Karunanayake, H. Wickramasinghe, N. Dikmadugoda, G. Premawansa, R. Wickramasinghe, H. J. de Silva, S. Premawansa, S. Rajapakse & S. Handunnetti (2015) Diagnosis of Leptospirosis: Comparison between Microscopic Agglutination Test, IgM-ELISA and IgM Rapid Immunochromatography Test. *Plos One,* 10.

Obrenovic, S., S. Radojicic, N. Stevic, D. Bogunovic, S. Vakanjac & M. Valcic (2014) Seroprevalence of cat leptospirosis in Belgrade (Serbia). *Acta Veterinaria-Beograd,* 64**,** 510-518.

Paepe, D. & S. Daminet (2013) Feline CKD: Diagnosis, staging and screening - what is recommended? *J Feline Med Surg,* 15 Suppl 1**,** 15-27.

Palaniappan, R. U. M., Y. F. Chang, C. F. Chang, M. J. Pan, C. W. Yang, P. Harpending, S. P. McDonough, E. Dubovi, T. Divers, J. X. Qu & B. Roe (2005) Evaluation of lig-based conventional and real time PCR for the detection of pathogenic leptospires. *Molecular and Cellular Probes,* 19**,** 111-117.

Palmirotta, R., G. Ludovici, M. L. De Marchis, A. Savonarola, B. Leone, A. Spila, F. De Angelis, D. Della Morte, P. Ferroni & F. Guadagni (2011) Preanalytical Procedures for DNA Studies: The Experience of the Interinstitutional Multidisciplinary BioBank (BioBIM). *Biopreservation and Biobanking,* 9**,** 35-45.

Papich, M. G. 2005. Drugs and the kidneys: Preventing and managaing their potential adverse effects. In *North American Veterinary Conference*, 857-860. Orlando, Florida: IVIS.

Parma, A. E., A. Seijo, P. M. Lucchesi, B. Deodato & M. E. Sanz (1997) Differentiation of pathogenic and non-pathogenic leptospires by means of the polymerase chain reaction. *Revista do Instituto de Medicina Tropical de Sao Paulo,* 39**,** 203-7.

Peterson, M. E. & D. A. Gamble (1990) Effect of nonthyroidal illness on serum thyroxine concentrations in cats – 494 cases (1988). *Journal of the American Veterinary Medical Association,* 197**,** 1203-1208.

Picardeau, M. (2013) Diagnosis and epidemiology of leptospirosis. *Medecine Et Maladies Infectieuses,* 43**,** 1-9.

Picardeau, M., E. Bertherat, M. Jancloes, A. N. Skouloudis, K. Durski & R. A. Hartskeerl (2014) Rapid tests for diagnosis of leptospirosis: Current tools and emerging technologies. *Diagnostic Microbiology and Infectious Disease,* 78**,** 1-8.

Poli, A., F. Abramo, D. Matteucci, F. Baldinotti, M. Pistello, S. Lombardi, P. Barsotti & M. Bendinelli (1995) Renal involvement in feline immunodeficiency virus-infection -P24 antigen-detection, virus isolation and PCR analysis. *Veterinary Immunology and Immunopathology,* 46**,** 13-20.

Poli, A., N. Tozon, G. Guidi & M. Pistello (2012) Renal Alterations in Feline Immunodeficiency Virus (FIV)-Infected Cats: A Natural Model of Lentivirus-Induced Renal Disease Changes. *Viruses-Basel,* 4**,** 1372-1389.

Polzin, D. J. (2013) Evidence-based step-wise approach to managing chronic kidney disease in dogs and cats. *Journal of Veterinary Emergency and Critical Care,* 23**,** 205-215.

Prescott, J. F., B. McEwen, J. Taylor, J. P. Woods, A. Abrams-Ogg & B. Wilcock (2002) Resurgence of leptospirosis in dogs in Ontario: recent findings. *Canadian Veterinary Journal-Revue Veterinaire Canadienne,* 43**,** 955-961.

Quimby, J. M. (2015) Feline chronic kidney disease: Understanding pathophysiology is the key to development of new treatments. *Veterinary Journal,* 203**,** 265-266.

Ravin, K. A. & M. Loy (2016) The Eosinophil in Infection. *Clinical Reviews in Allergy & Immunology,* 50**,** 214-227.

Rees, H. G. (1964) Leptospirosis in a cat. *New Zealand Veterinary Journal,* 12**,** 64.

Reilly, G. A. C., N. C. Bailie, W. T. Morrow, S. W. J. McDowell & W. A. Ellis (1994) Feline stillbirths associated with mixed salmonella-typhurium and leptospira infection. *Veterinary Record,* 135**,** 608-608.

Reitstetter, R. E. (2006) Development of species-specific PCR primer sets for the detection of Leptospira. *Fems Microbiology Letters,* 264**,** 31-39.

Reynolds, B. S. & H. P. Lefebvre (2013) Feline CKD: Pathophysiology and risk factors--what do we know? *J Feline Med Surg,* 15 Suppl 1**,** 3-14.

Rodriguez, J., M. C. Blais, C. Lapointe, J. Arsenault, L. Carioto & J. Harel (2014) Serologic and Urinary PCR Survey of Leptospirosis in Healthy Cats and in Cats with Kidney Disease. *Journal of Veterinary Internal Medicine,* 28**,** 284-293.

Ronco, C., J. A. Kellum & M. Haase (2012) Subclinical AKI is still AKI. *Critical Care,* 16**,** 4.

Ross, L. (2011) Acute Kidney Injury in Dogs and Cats. *Veterinary Clinics of North America-Small Animal Practice,* 41**,** 1-14.

Ross, S. J., C. A. Osborne, C. Lekcharoensuk, L. A. Koehler & D. J. Polzin (2007) A case-control study of the effects of nephrolithiasis in cats with chronic kidney disease. *Javma-Journal of the American Veterinary Medical Association,* 230**,** 1854-1859.

Roudebush, P., D. J. Polzin, S. J. Ross, T. L. Towell, L. G. Adams & S. Dru Forrester (2009) Therapies for feline chronic kidney disease. What is the evidence? *J Feline Med Surg,* 11**,** 195-210.

Ruhl-Fehlert, C. I., S. Brem, W. Feller, H. Kopp, P. Meyer & M. Rinke (2000) Clinical, microbiological and pathological observations in laboratory beagle dogs infected with leptospires of the serogroup Sejroe. *Experimental and Toxicologic Pathology,* 52**,** 201-207.

Schuller, S., T. Francey, K. Hartmann, M. Hugonnard, B. Kohn, J. E. Nally & J. Sykes (2015) European consensus statement on leptospirosis in dogs and cats. *Journal of Small Animal Practice,* 56**,** 159-179.

Shang, E. S., T. A. Summers & D. A. Haake (1996) Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic Leptospira species. *Infection and Immunity,* 64**,** 2322-2330.

Sharma, K. K. & U. Kalawat (2008) Early diagnosis of leptospirosis by conventional methods: One-year prospective study. *Indian Journal of Pathology and Microbiology,* 51**,** 209-211.

Shropshire, S. B., J. K. Veir, A. K. Morris & M. R. Lappin (2015) Evaluation of the Leptospira species microscopic agglutination test in experimentally vaccinated cats and Leptospira species seropositivity in aged azotemic client-owned cats. *J Feline Med Surg*. 18, 768-772

Siracusa, M. C., B. S. Kim, J. M. Spergel & D. Artis (2013) Basophils and allergic inflammation. *Journal of Allergy and Clinical Immunology,* 132**,** 789-801.

Slack, A. T., M. L. Symonds, M. F. Dohnt & L. D. Smythe (2006) Identification of pathogenic Leptospira species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B encoding gene. *BMC Microbiology,* 6**,** 95.

Sohail, M. L., M. S. Khan, M. Ijazi, M. Avais, M. Y. Zahoor, O. Naseer & M. U. Saleem (2017) Evidence of Clinicopathological Changes during Equine Leptospirosis. *Pakistan Journal of Zoology,* 49**,** 849-853.

Sparkes, A. H., R. Heiene, B. D. X. Lascelles, R. Malik, L. R. Sampietro, S. Robertson, M. Scherk & P. Taylor (2010) ISFM and AAFP concensus guidelines: Long-term use of NSAIDs in cats. *Journal of Feline Medicine and Surgery,* 12**,** 521-538.

Stoddard, R. A., J. E. Gee, P. P. Wilkins, K. McCaustland & A. R. Hoffmaster (2009) Detection of pathogenic Leptospira spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. *Diagnostic Microbiology and Infectious Disease,* 64**,** 247-255.

Stokes, J. E. & S. D. Forrester (2004) New and unusual causes of acute renal failure in dogs and cats. *Veterinary Clinics of North America-Small Animal Practice,* 34**,** 909-922.

Sykes, J. E., K. Hartmann, K. F. Lunn, G. E. Moore, R. A. Stoddard & R. E. Goldstein (2011) 2010 ACVIM Small Animal Consensus Statement on Leptospirosis: Diagnosis, Epidemiology, Treatment, and Prevention. *Journal of Veterinary Internal Medicine,* 25**,** 1-13.

Tansuphasiri, U., R. Chanthadee, D. Phulsuksombati & N. Sangjun (2006) Development of a duplex-polymerase chain reaction for rapid detection of pathogenic Leptospira. *Southeast Asian Journal of Tropical Medicine & Public Health,* 37**,** 297-308.

Thaipadunpanit, J., W. Chierakul, V. Wuthiekanun, D. Limmathurotsakul, P. Amornchai, S. Boonslip, L. D. Smythe, R. Limpaiboon, A. R. Hoffmaster, N. P. J. Day & S. J. Peacock (2011) Diagnostic Accuracy of Real-Time PCR Assays Targeting 16S rRNA and lipl32 Genes for Human Leptospirosis in Thailand: A Case-Control Study. *Plos One,* 6**,** 6.

Townsend, W. M., J. Stiles & S. G. Krohne (2006) Leptospirosis and panuveitis in a dog. *Veterinary Ophthalmology,* 9**,** 169-173.

Toyokawa, T., M. Ohnishi & N. Koizumi (2011) Diagnosis of acute leptospirosis. *Expert Rev Anti Infect Ther,* 9**,** 111-21.

Trivedi, S. V., A. H. Vasava, L. C. Bhatia, T. C. Patel, N. K. Patel & N. T. Patel (2010) Plasma exchange with immunosuppression in pulmonary alveolar haemorrhage due to leptospirosis. *Indian Journal of Medical Research,* 131**,** 429-433.

Tulsiani, S. M., G. C. Graham, M. F. Dohnt, M. A. Burns & S. B. Craig (2011) Maximizing the chances of detecting pathogenic leptospires in mammals: the evaluation of field samples and a multi-sample-per-mammal, multi-test approach. *Annals of Tropical Medicine and Parasitology,* 105**,** 145-162.

Ullmann, L. S., J. L. Hoffmann, W. de Moraes, Z. S. Cubas, L. C. dos Santos, R. C. da Silva, N. Moreira, A. M. S. Guimaraes, L. G. Camossi, H. Langoni & A. W. Biondo (2012) Serologic survey for

leptospira spp. In captive neotropical felids in Foz do Iguaco, Parana, Brazil. *Journal of Zoo and Wildlife Medicine,* 43**,** 223-228.

Vaden, S. L., J. Levine & E. B. Breitschwerdt (1997) A retrospective case-control of acute renal failure in 99 dogs. *Journal of Veterinary Internal Medicine,* 11**,** 58-64.

van de Maele, I., A. Claus, F. Haesebrouck & S. Daminet (2008) Leptospirosis in dogs: a review with emphasis on clinical aspects. *Veterinary Record,* 163**,** 409-413.

van de Werve, C., A. Perignon, S. Jaureguiberry, F. Bricaire, P. Bourhy & E. Caumes (2013) Travel-Related Leptospirosis: A Series of 15 Imported Cases. *Journal of Travel Medicine,* 20**,** 228-231.

van Hoek, I., H. P. Lefebvre, K. Peremans, E. Meyer, S. Croubels, E. Vandermeulen, H. Kooistra, J. H. Saunders, D. Binst & S. Daminet (2009) Short- and long-term follow-up of glomerular and tubular renal markers of kidney function in hyperthyroid cats after treatment with radioiodine. *Domestic Animal Endocrinology,* 36**,** 45-56.

Vaske, H. H., T. Schermerhorn & G. F. Grauer (2016) Effects of feline hyperthyroidism on kidney function: a review. *Journal of Feline Medicine and Surgery,* 18**,** 55-59.

Wakeling, J., K. Moore, J. Elliott & H. Syme (2008) Diagnosis of hyperthyroidism in cats with mild chronic kidney disease. *Journal of Small Animal Practice,* 49**,** 287-294.

Watson, A. D. J. (1998) Urine specific gravity in practice. *Australian Veterinary Journal,* 76**,** 392-398.

White, J. D., R. Malik, J. M. Norris & N. Malikides (2010) Association between naturally occurring chronic kidney disease and feline immunodeficiency virus infection status in cats. *Javma-Journal of the American Veterinary Medical Association,* 236**,** 424-429.

Williams, T. L. & J. Archer (2016) Evaluation of urinary biomarkers for azotaemic chronic kidney disease in cats. *Journal of Small Animal Practice,* 57**,** 122-129.

Williams, T. L., J. Elliott & H. M. Syme (2014) Association between urinary vascular endothelial growth factor excretion and chronic kidney disease in hyperthyroid cats. *Research in Veterinary Science,* 96**,** 436-441.

Williams, T. L., K. J. Peak, D. Brodbelt, J. Elliott & H. M. Syme (2010) Survival and the Development of Azotemia after Treatment of Hyperthyroid Cats. *Journal of Veterinary Internal Medicine,* 24**,** 863-869.

Yang, C. W., M. S. Wu, M. J. Pan, W. J. Hsieh, A. Vandewalle & C. C. Huang (2002) The Leptospira outer membrane protein LipL32 induces tubulointerstitial nephritis-mediated gene expression in mouse proximal tubule cells. *Journal of the American Society of Nephrology,* 13**,** 2037-2045.